LABORATORY MANUAL

PRATICAL VETERINARY MICROBIOLOGY

VMC- Unit-1

Course title – General and systematic Veterinary Bacteriology

Name – ……..

Roll No / Enroll No -……/……

Batch -……..

Session –……..

Department of Veterinary Microbiology
Mahatma Jyotiba Fule College of Veterinary & Animal Science
Chomu, Jaipur

CERTIFICATE

This is to be certify that

Mr./Ms. …… …… …… ……..……

Roll. No…… …… … ……of Second year B.V.Sc& A.H.

has successfully completed all practical's in Veterinary Microbiology during

Third year of the academic year ……

Date :

Place : CHOMU, JAIPUR

Head of Department **Course Teacher**

PREFACE

This Laboratory Manual has been prepared for the undergraduate students of B.V.Sc. & A.H. in accordance with the syllabus designed by the Veterinary Council of India. The efforts have been made to make the manuscript worthy, realistic and easily understandable for the students, teachers and Veterinary Practitioners for diagnosis of different microbial diseases of animals. We hope this manual will serve very useful tool to the undergraduate and graduate students of Veterinary Science who are undergoing courses in veterinary microbiology.

It‟s our pleasure to thank Dean Sir, M.J.F College of veterinary and Animal Sciences, Chomu, Jaipur for providing necessary facilities and rendering all helps in preparing this course manual.

Computer operator and typist Mr. Ashutosh Sharma worked hard for very existence of this manual so I acknowledge his efforts.

Course Incharge
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EX.1 GENERAL LABORATORY INSTRUCTIONS

1. Put on clean laboratory apron while working in the laboratory. Place only those materials needed for the day"s laboratory exercise on the bench tops. Purses, coats extra books, etc. should be placed in the lab bench storage areas or under the lab benches in order to avoid damager or contamination.
2. Wash your hands and tabletop before and after each laboratory period with some antiseptic lotion, or soap.
3. Never touch inoculation loop, cotton plugs or any other similar material to your lips. Smoking in the laboratory in strictly prohibited.
4. If a culture is spilled, cover the area with antiseptic lotion and inform the teacher for its proper disposal.
5. Mouth pipetting is not permitted under any circumstances. Use the safety pipetting devices which are provided.
6. Sterilize the inoculation loop before and after its use.
7. Place discarded cultures on the assigned place.
8. Place used cotton, filter papers and similar other discarded material in the wastebasket. Do not leave them on the table or throw on the floor.
9. Label all your cultures properly before placing them in the incubator. They should always be place on the assigned place in the incubator.
10. When not in use, put off gas burner and microscope lamp.
11. Always use the assigned microscope that should be kept properly cleaned. Use tissue papers for cleaning lenses.
12. All reagents, stains and other equipment should be returned to their original places at the end of the laboratory period.
13. While leaving laboratory see that gas, electric and water points are off.
14. Always, at the end of each laboratory period, carefully clean oil from the objective and condenser lenses, align the low power dry objective with the condenser and rack condenser up and body tube down.
15. Always place culture tubes of broth or slants in an upright position in a rack. Do not lay them down on the table or lean them on other objects. They may roll onto the floor and break.
16. All culture containers which are to be incubated should bear the following notations:1) initials (or last name of the student) 2) specimen (name of organism or number of unknown) 3) date. When using Petri plates, these notations should be entered on the bottom half, not the lid. Unless otherwise directed, all plates are to be inverted, all plugged tubes should have the plugs firmly set into the tubes, and all screw cap tubes should have the caps loosened one- half turn to permit gas exchange.

EX – 2 STERILIZATION & DISINFECTION

Ex 2.1 STERILIZATION BY HEAT

For bacteriological studies it is necessary to deal with the pure cultures of bacteria. As under normal circumstances bacteria are present in almost all situations exposed to the atmosphere, it is desirable to eliminate the existing bacterial flora from unsterilized media and the surfaces of the glassware. The sterilized media and other materials are prepared and kept in such a way that their sterility is maintained throughout their use.

The destruction of microorganisms may be accomplished by physical or chemical means. Under physical methods of sterilization, hear (dry or moist) and filtration are most commonly used. In this exercise sterilization by hear will be demonstrated.

A. STERILIZATION BY DRY HEAT

(A) Hot air oven

Certain materials like inoculation loop, spatula, mouth of test, tubes, etc., can be sterilized directly on the flame of the Bunsen burner. Hot air oven which may be gas or electrically operated and circulates hot air is very commonly used equipment in the bacteriological laboratories. The materials which can be sterilized by hot air oven are glassware, all glass syringes, powders, fats and oils. Before sterilization glassware must be dry and properly wrapped in papers. Serological pipettes may be sterilized in metal cases. Flasks and test tubes should be properly stoppered with non-absorbent cotton wool. Since the heat distribution is less effective in this apparatus, it is customary to apply a temperature of 160°C to 180° C for at least one hour. A temperature of 180°C , if prolonged will char or even burn paper or cotton and cause discoloration of glassware. The students should know the construction and working of the equipment.

Materials

Hot air oven and various types of glassware for sterilization.

Procedure

1. Wrap the glassware in paper.
2. Place the material inside the apparatus. Do not over- pack the space. Close the door and allow the equipment to function.
3. Maintain the temperature at 170°C for 1 hour and disconnect the electric connection.
4. Allow the temperature to come down and take out the material.

B. STERILIZATION BY MOIST HEAT

(A) **Serum inspissator** - Inspissation is a procedure of fractional sterilization by moist heat at lower temperature. In this method temperature of 80° C for 2 hours on

three successive days in satisfactory. The students should be made aware of the construction of the equipment.

Materials - Serum inspissator and serum or egg media for sterilization

Procedure

1. Place test tubes containing about 8 ml medium in a slanting position so as to form thick slants after coagulation.
2. Put on the electric switch and maintain the temperature of 80°C for 2 hours.
3. Repeat process of sterilization for 3 consecutive days. The medium should be incubated at 37°C in between the sterilization timings.

(**B) Arnold sterilizer** - A temperature higher than 100°C will affect media and solutions (e.g., sugar media) adversely. Sterilization at 100°C can be accomplished in Arnold sterilizer with free flowing steam. Since heat will not destroy all spores at 100° C the method of fractional sterilization (tyndallization) must be used for complete sterilization. This consists of heating the media to a temperature of 100°C for approximately 30 minutes for three successive days. This process permits the resistant spores to germinate during the intermittent incubation periods between two successive sterilizations. An autoclave with free flowing steam may also be used for fractional sterilization if Arnold sterilizer is not available. The construction of the apparatus should be explained to the students during the demonstration period.

Materials - Arnold sterilizer and media for sterilization.

Procedure

1. Place the media in the inner compartment of the apparatus and close the lid.
2. Bring the water to boil and maintain the boiling temperature of 30 minutes.
3. Remove the media and incubate at 37°C for over night.
4. Repeat the process No. 2 and 3 on second and third days before using media.

(C) **Instrument sterilizer** - Certain instruments like forceps, scissors, syringes, needles, etc. can be sterilized in an instrument sterilized. Boiling the equipment in water for about 15 minutes will kill all non-sporing and many sporing organisms. This method of sterilization has only a limited use in bacteriological laboratory.

Materials - Electric or gas heated instrument sterilizer and materials to be sterilized.

Procedure

1. Prepare the instruments for sterilization and keep them in the trays of the sterilizer. The instrument should be submerged in the water.
2. Boil the water for about 15 minutes and take out the instruments when temperature goes down.

(D) Autoclave - The most effective and reliable method of sterilization is by steam under pressure which is accomplished by an autoclave It is usually sufficient to expose the material for 15mintes at 15 lb pressure. This will accomplish a temperature of 121°C and will kill all the bacteria and their spores. Water boils at 100°C at atmospheric pressure. As the pressure is increased, the temperature becomes higher On the other hand if the pressure is decreased the boiling temperature is reduced (boiling point at 0 attitude is 100°C while at higher attitude it is less than 100°C). Steam sterilization is more effective than dry heat because steam easily penetrates to all parts of the vessel. Moist heat denatures protein at a lower temperature than dry heat. Most of the bacteriological media, aprons, bandages, surgical instruments, syringes, rubber appliances, discarded cultures, etc. are safely and effectively sterilized by steam under pressure. Sugars fats and oils cannot be sterilized in autoclave at usual pressure and temperature. The construction and the principle of the working of the autoclave should be explained to the students.

Materials - Autoclave and materials for sterilization

Procedure

1. Place the material in the sterilization chamber of the autoclave. Close the lid and put on the electric connection.
2. Open steam escape valve till steam escapes freely from the steam chamber.
3. Close steam valve and allow pressure to rise slowly until the desired pressure and temperature is reached.
4. Maintain the desired pressure and temperature for 20 minutes and put off the electric connection
5. Open steam valve and permit the autoclave to cool down until the pressure gauge reads zero.
6. Open the door of the sterilizing chamber and remove the sterilized material.

EX. 2.2 STERILIZATION BY FILTRATION

Media like urea, blood serum, sugar, etc., are affected by heating at temperatures normally employed for sterilizing other materials. Such heat labile liquid media can be freed from bacteria by filtration. The filters remove bacteria either by mechanically withholding them from passing through the minute pores of the filter or by absorption of the bacteria to the filter because of the difference in their electrical charges

The commonly used microbiological filters are:

(a) Sintered glass filters: These are made of fused porous glass.
(b) Seitz filters: These are made of asbestos pads.
(c) Chamberland and selas candle type filters: These are made of unglazed porcelain.
(d) Berkefeld and mandler filters: These are made a diatomaceous earth.
(e) Membrane filters: These are made of cellulose membrane.

All the filter mentioned above are used by attaching them to a vacuum pump. In this exercise the working of Seitz filter will be demonstrated to the students. The principle of working of other types of filters should also be explained by the instructor.

Materials - Seitz filter assembly, vacuum and pressure pump and the liquid for filtration.

Procedure

1. Connect the vacuum and pressure pump with the side nozzle of a safety device which in its turn is connected to the Erlenmeyer flask of the filter assembly
2. Pour liquid in the funnel of the filter and let the vacuum and pressure pump function by means of negative pressure the liquid will pass through the asbestos filter pad into the empty flask which will be bacteria- free.

Ex .2.3 STERILIZATION BY CHEMICAL AGENTS

Many chemicals are toxic to microorganisms. The chemical agents inhibit cell activities and its growth (antiseptics) or are lethal to microorganisms (disinfectants) The properties of an ideal disinfectant are toxicity to microorganisms, non –corroding and non-staining, non unpleasant odour, moderate cost and easy availability. The major disinfectants are:

1. Phenol and phenolic compounds: They act by denaturing the proteins and by damaging the cell membrane.
2. Alcohols : they act as protein coagulant and are used frequently as skin disinfectants most effective strength of alcohol is 70%
3. Iodine: This is chiefly used as skin disinfectant.
4. Chlorine and chlorine compounds: Free chlorine gas is difficult to handle. Hypochlorites (calcium and sodium compounds) available in powder or liquid forms are commonly used as disinfectants and sanitizing agents. Chloramines, another category of chlorine compounds are more stable.
5. Heavy metals and their compounds: Mercury, silver and copper or their compounds generally act by combining with SH group of enzymes.
6. Detergents: Detergents are surface tension depressants or wetting agents and are primarily used for cleansing surfaces. They damage cell membrane. Detergents are anionic (e.g. soap) as well as cationic (e.g. quaternary ammonium compounds) somewhat less effective against gram negative bacteria and fungi. They are widely used as skin antiseptics and sanitizing agents.
7. Oxidizing agents: Hydrogen peroxide and potassium permanganate are mildly antiseptic. They are less stable,
8. Acids and alkalies: The killing action of mineral acid (HCL, H_2SO_4) which are not commonly used as disinfectants, is based on dissociation and final H^+ ion

concentration. Organic acids (acetic acid, benzoic acid) act differently as they ionize to a relatively low degree. They are generally used as preservative for food. Alkalies (e.g NaOH) dissociate to OH ions and are more effective against gram negative bacteria and viruses. Acid fast organisms are resistant to alkali treatment. The antiformin treatment of tuberculous material is based on theis principle before culturing.

9. Gaseous sterilization (aerosols): Formaldehyde is most effective in 37% aqueous solution. This is generally used for fumigation of egg incubators, eggs and poultry houses, It acts best at 22°C with a humidity of 60 to 80% Another agent, ethylene oxide, is reliable substance for gaseous sterilization of dry surfaces.
   In this exercise, egg incubator will be sterilized by fumigation.

Materials - Egg incubator, formalin, potassium and a bucket.

Procedure

1. Calculate the air space of the incubator in terms of cubic feet.
2. Add sufficient quantity of potassium permanganate in the bucked and then pour sufficient quantity of formalin on it (250 gm of $KMnO_4$ and 500 ml of formalin are sufficient to fumigate 1000 cu. ft. space).
3. Immediately place the bucket inside the incubator and close the door air-tight
4. After 24 hours of fumigation. Open the door of the incubator and remove the bucket of $KMnO_4$ and formalin. Within a short period, the incubator will be cleared of the fumes.

EX.2.4 PHENOL COEFFICIENT TEST

Phenol coefficient test has been accepted for evaluation of a disinfectant. The phenol coefficient of a disinfectant is the highest dilution of the disinfectant divided by the highest dilution of phenol which will kill the test organism in 10 minutes but not in 5 minutes under standard conditions. Specific strain of *Salmonella typhosa* or *Staphylococcus aureus* is used as the test organism.

Materials

10 ml nutrient broth tubes 30, sterile 1 ml pipettes 5, sterile 10 ml pipette 1, inoculation loop 4 mm diameter 1, 10 ml 24 hours broth culture of *Staph. Aureus,*5 ml each of 1/80, 1/90, and 1/100, dilutions of phenol and 5 ml each of 1/100, 1/150, 1/200, 1/250, 1/300, 1/350, 1/400, dilutions of disinfectant under tets.

Procedure

1. Place 10 tubes, i.e 3 tubes containing 5 ml of different dilutions of phenol (1/80,1/90 and 1/100) and 7 tubes containing 5 ml of different dilutions of test

disinfectant (1/100, 1/150, 1/200,1/250, 1/300, 1/350, and 1/400) in the first row of the tube rack.

2. In each of the second, third and fourth rows of the rack , place 10 tubes containing 10 ml broth behind dilutions of phenol and test disinfectants.
3. Label the broth tubes in the second row as 5 min. in the third row as 10 min. and in the fourth row as 15 min.
4. At 30 second intervals successively inoculate each of the dilutions of the phenol and test disinfectant (in the first row) with 0.5 ml culture of *Staph. aureus.* This will take total of 4.5 minutes
5. After 30 seconds of inoculation of the last tube of disinfectant, transfer 1 loopful of the material form the first tube of the phenol dilution to the first broth tube in 5 min row And then at 30 second intervals continue to transfer 1 loopful of the culture from phenol/test disinfectant tubes to corresponding broth tubes of 5 min.row. After finishing 5 minutes allow 30 seconds to pass and proceed to transfer 1 loopful of the material from phenol and test disinfectant dilutions to the corresponding broth tubes of 10 min and 15 min in the same manner.
6. After completing the last inoculation, place the rack in incubator at 37°C and observe.

Results

Records the results in the chart as (+) if there is growth and (-) if there is no growth and calculate the phenol coefficient of the disinfeciant.

No of row	**Exprosure time**	**Phenol dilution**			**Test disinfectant dilution**						
1st row (5 ml disinfectant)	-	1/80	1/90	1/100	1/100	1/150	1/200	1/250	1/300	1/350	1/400
2nd row (10 ml broth)	5 min.										
3rd row (10 ml broth)	10 min.										
4th row (10 ml broth)	15 min.										

The phenol coefficient = $\frac{\text{Highest dilution of the disinfectant (killing in 10 min.but not in 5 min)}}{\text{Highest dilution of phenol (killing in10 min but not in5 min)}}$

EX. 3 MOTILITY OF BACTERIA BY HANGING DROP METHOD

A) HANGING DROP AND CAVITY SLIDE

Materials: Young broth cultured *Proteus vulgaris,* concavity slides cover slips wire loop, petroleum jelly, and microscope.

Procedure:

1. Apply some petroleum jelly either on the four corners of cover slip or around the depression of the concavity slide.
2. Place a loopful of culture suspension on the cover slip.
3. Invert the concavity slide over the cover slip in such away that the drop must come in the centre of the depression.
4. Quickly, but carefully, turn the slide upside down so that the cover slip is uppermost producing hanging drop of the cover slip.
5. Place the slide on the stage of the microscope and observe under low power objective. Forcus the periphery of the cover clip.
6. Turn the high power dry objective into the position and observe the motility with lowered condenser.

Results: Observe the motility and record the results.

Note:

1. The culture must be young.
2. Obtain the best illumination by adjusting the condenser diaphragm and by raising or lowering the condenser. Failure to detect the organism is usually a result of excessive illumination. To increase the contrast when examining living cells reduce the light intensity by closing the diaphragm. It is help to focus first on the edge of the drop and bacteria will often concentrate is this region.
3. The distinction between true motility and Brownian movement, which is undergone by all small objects suspended in water, is not readily made without experience. Perhaps the best criterion of true motility is that the cell should move through a distance several times greater than its own length with cells moving in different directions slide, Many cells of a culture should be carefully observed before the conclusion that the culture is non- motile is reached.
4. Immediately after examination the cover slip and slide should be separated from one another and both dropped in disinfectant.
5. Obligate anaerobes must be observed under anaerobic conditions, i.e. in a capillary tube.

Ex. 4 -MEDIA PREPERTION & ROUTINE CULTURE MEDIA

Ex 4.1- PREPARATION OF CULTURE MEDIA

Most culture media are available commercially as readymade dehydrated form. It is less costly to use readymade media, since the ingredients are often required in small amounts, but available in large quantities if purchased. Some of the chemicals are also difficult to obtain.

To ensure good performance and reproducibility in the results the following must be performed correctly.

- Weighing and dissolving of the ingredients.
- Addition of heat sensitive material.
- P^H testing.
- Dispensing and sterilization.
- Sterility testing and quality control.
- Storage.

Addition of heat sensitive ingredients

If the ingredient has been refrigerated, for example blood or serum, if must be allowed to warm to room temperature before being added to a molten agar medium. The heat sensitive ingredient should be added when the medium has cooled to about 50^o C. An aseptic technique must be used.

p^H of medium

Microorganisms are sensitive in varying degrees to the p^H of the external environment. Although this is important for survival, it is even more important for growth, where there is an optimal for the growth of the organism concerned. Most pathogenic bacteria have a fairly restricted p^H range and grow best around p^H 7.3 i.e., at a slightly alkaline reaction. This may be reflection of the fact that the pH of mammalian blood and tissues is of this order. On the other hand, commensal and saprophytic bacteria often have a wider p^H growth range. Yeasts and fungi generally have an acid optimum. Two types of method are generally employed for the measurement of p^H in the laboratory. These depend either upon the use of electric p^H meters or upon pH indicator dyes.

The p^H meter-

The only accurate method of measuring p^H is with a p^H meter, and in laboratories where numerous determination p^H are required; this apparatus is a necessary piece of equipment. It is easy and quick to use although care must be taken in its maintenance.

The p^H indicator dyes-

Indicator dyes are substances that will changes in colour with variations in the p^H of the solution in which they are dissolved. For example, phenol sulpha naphthalene

(phenol red) is yellow in acid solution and red in alkaline solution. If alkali is gradually added to an acid solution containing phenol red, have different colours for different p^H values and this can be used to determine p^H. It must be emphasized that outside the range at which the colour is changing, an indicator can show only whether the solution is more acid or more alkaline than the indicator range

P^H papers

The simplest method of determining the p^H of a solution is to use commercially available pH indicator papers. These papers are impregnated with an indicator that gives a change of colour over a specific or general range of p^H. The paper can simply be dipped in the solution to be tested or, alternatively, a drop of the solution can be withdrawn by a wire loop or Pasteur pipette and placed on the paper. The resulting colur is compared with the chart supplied with the paper. Sets of wide range and narrow range p^H papers are available from several suppliers, e.g., Whatman, BDH, Merck, Ranbaxy, etc.

An agar medium can be tested by pouring a sample of the molten medium into a small beaker or petri disk and when it has solidified, layin a narrow range p^H paper on its surface. The colour of the paper is then compared against the p^H colour chart provided.

Colorimetric measurement of p^H

It can also be made with a simple instrument called p^H comparator set. This procedure is based on the fact that some compounds (acid-base indicators) very in colour within certain limits of p^H. Thus by selective of a selective of a suitable indicator, the p^H of a medium can be determined. Standard colour discs of different colour indicators are available which show the colour transition over a p^H range.

Adjustment of p^H- The p^H of a dehydrated medium should be require adjustment providing it has been prepared correctly using pure water and clean equipment, and it has not been over autoclaved. The manufacture‟s instructions must be followed exactly.

The p^H of other media should be adjusted as directed in the method of preparation adjustment should be carried out suing N/10 sodium hydroxide if the medium is too acid, and N/10 hydrochloric acid if too alkaline.

When adjusting the p^H of a large volume of media it is best to measure the amount of acid or alkali that needs to be added to adjust 10 ml of the medium and then calculate the amount required to adjust the remaining volume.

Note: - It is better to have a medium slightly alkaline rather than slightly acid so that acid, and not alkali needs to be added. If an alkali is added to an acid medium phosphates will be precipitated and the medium will require filtering.

Table 4.1 P^H indicators

Indicator	Range of pH	Colour change
Thymol blue (acid range)	1.2-2.8	Red to yellow
Bromophenol blue	2.8-4.6	Yellow to violet
Bromocresol green	3.6-5.2	Yellow to blue
Methyl red	4.4-6.2	Red to yellow
Litmus	4.5-8.3	Red to blue
Bromocresol purple	5.2-6.8	Yellow to violet
Bromothymol blue	6.8-7.6	Yellow to blue
Neutral red	6.8-8.0	Red to yellow
Phenol red	6.8-8.4	Yello to purple-pink
Cresol red	7.2-8.8	Yellow to violet-red
Thymol blue(alkaline range)	8.0-9.6	Yellow to blue
Phenolphthalein	8.3-10.0	Colourless to red
Thymolphathalein	9.3-10.5	Colourless to blue
BDH Universal	3.0-11.0	Red-orange-blue-reddish voilet

Distribution of unsterilized media

In general, media are initially tubed or bottled without sterile precaution. All media are distributed as liquids. Sophisticated dispensing machines are commercially available and are recommended for busy laboratories.

Liquid media may be distributed in test tubes or bottles. In general, a test tube is half filled. Small screw- capped bottles in the range 5-30 ml may be safety autoclaved with the caps tight. Larger bottles (more than 50 ml capacity) should not be filled more than 75-80% full. Solid media are generally prepared by the addition of agar or gelatin in powder form with initial heating and mixing to ensure even dispersal of the ingredients. Distribution of the molten medium may be done at this stage as for liquid media, with careful precautions. Thereafter, the said media are sterilized by autoclaving.

Sterilizing culture media

Always sterilize a medium at the correct temperature and for the correct length of time as instructed in method of preparation. If using a dehydrated medium, always follow exactly the manufacturer‟s instructions.

The methods commonly used to sterilized culture media are:

- Autoclaving
- Steaming at 100°C: and
- Filtration

Autoclaving – Autoclaving is used to sterilize most agar and fluid culture media.

Table 4.2 sterilization times at 121°C

Volume of medium	Container	
	Flask	Bottle
10 ml	15 min	20 min
100 ml	20 min	25 min
500 ml	25 min	30 min
1 liter	30 min	40 min

Tubes and bottles of medium must be put in the autoclave such that has free access to them. They must not to be held in airtight tins. Wire crates are suitable.

Sometimes lower temperatures, such as 115°C for times ranging from 10-20 min are recommended for sterilization of media containing ingredients that are not very stable to heat. These conditions are not strictly reliable for sterilization and should be used only for media distributed in small quantities. They are usually satisfactory because it is unlikely that many heat resistant spores would be present in media prepared under clean conditions.

Steaming at 100°C : This is used t sterilize media containing ingredients that would be broken down or inactivated at temperatures over 100°C Examples include selenite F enrichment medium and Cary-Blair transport medium. Steaming is also used to remelt previously bottled sterile agar media.

Steaming can be performed in an autoclave with the lid left loose, or in any form of steam sterilizer such as an Arnold of Koch steamer. The bottle of media with loosened caps is placed on perforated trays above the boiling water. After sterilization, when medium, has cooled, the bottle tops are tightened. Steaming time varies according to the type of medium, for example 10 min is recommended for selenite medium and 15 min for Cary Blair medium.

Occasionally, a form of steaming called tyndalization is used to sterilize media. It consists of steaming on three successive days. On the first day the medium is steamed for 90 to 120 min to kill the vegetative cells. It is then left overnight in on incubator to encourage the germinatin of endospores. On the second and third day the medium is steamed for a further 90-120 min to ensure the destruction of the germinated spores with intermittent incubation.

Filtration- This provides a means of removing bacteria from fluids. It is used mainly to sterilize additives that are heat sensitive and cannot be autoclaved, or less stable substances that need to be added to a sterile medium immediately before use, such as blood serum and solutions containing urea and certain carbohydrates.

Several different types of filters can be used, including those made form sintered glass, asbestos or inert cellulose esters. Cellulose filters are referred to as membrane filters. They are preferred to other types of filters because they filter more rapidly, do not affect the filtrate in any way, and adsorb very little of the substance being filtered.

Sterility testing

For media in screw-cap tubes or bottles, the simplest way to for contamination is to incubate the batch (or as many as possible from the batch) at 35-37°C overnight contamination by microorganisms capable of overnight growth will be shown by turbidity in a fluid medium add growth on or in a solid medium.

Note: All media, even those that have been sterility tested at the time of preparation, should always be checked visually for contamination immediately before being inoculated.

Distribution of sterilized media with sterile precaution

Liquid media: Sterile precautions for tubing and bottling liquid media are necessary if an ingredient of the medium is heat-labile for example certain sugars used in fermentation test media. The ingredients that are stable to heat are prepared and sterilized: the unstable ingredient (previously sterilized in a suitable way) is added with sterile precaution and the medium is distributed with sterile precautions into sterile containers.

Alternatively the heat- stable part of the medium may be distributed into clean glassware without sterile precautions and then sterilized. The sterile heat table ingredient can later be added aseptically from a sterile graduated pipette or syringe.

Solid media:- This may be distributed in test tubes or bottles, as for liquid media. The commonest shape is the slope or slant which provides a larger surface area of medium for inoculation for 150x16 mm test tubes, 5 ml of medium is sufficient and it is allowed to set at such an angle that there is a thick butt at the bottom. After cooling, fresh agar slopes contain water of condensation at the tube, which should not be allowed to run over the surface of the medium or wet the plug or cap.

If the medium is to be used for a "stab" or shake culture the test is half filled with the medium, which is allowed to solidify in the upright position. Screw capped bottles can be substituted for test tubes. The amounts of medium for slopes in 30 ml and 6 ml bottle are 5 ml and 2.5 ml, respectively. The medium may be allowed to set at an angle to form a butt as in test tubes, but it is easier to inoculate with a loop if it is parallel to the side of the bottle. For stab or shake culture, 30 ml bottles are half filled with medium.

Pouring plates:- Solid media in peteri dishes are usually referred to as "plates". For a dish of 90mm diameter 15 ml of medium is usually sufficient. Place are always pured with sterile precautions. Bulk medium is prepared sterile and is poured into steile petri dishes machines are commercially available for the automatic distribution of melted medium and for the automatic stacking of the prepared plates. The following points merit consideration for the automatic or manual pouring plates:

1. Avoid airborne contamination.
2. Work in a clean draught-free room preferably within an inoculation hood or cabinet with a filtered air supply.
3. To reduce condensation of water on the petri dish lids, the medium should be cooled to 50°C before pouring.
4. The melted medium should be poured into dishes on a flat surface and the dishes left undisturbed until the medium has set.
5. In separating organisms from mixed cultures by plating it is essential that the surface of the medium should be dry.

6. When plates have been poured the steam from the hot liquid condenses on top of the medium add this moisture is undesirable. It is removed by drying the poured and set plates in a warming or dying cabinet at 60°C for 5-30 min depending on the medium, with the plate inverted.

Basic method of preparation of medium

1. Weigh the solid ingredients by using an analytical balance.
2. Add sufficient quantity of distilled water.
3. Dissolve the ingredients by using a glass rod or by using a magnetic stirrer. If necessary heat the solution to room temperature.
4. Check pH by using and a narrow range pH paper and adjust required pH by using sodium hydroxide or hydrochloric acid.
5. Add agar and heat to dissolve.
6. If indicator is required. It is added after cooling the solution to about 40°C.
7. Final volume is made by using distilled water.
8. The prepared medium is distributed while hot, in the concial flasks, bottles and test tubes.
9. The medium in various containers sterilized by autoclaving at 121°C for 15 min.
10. The containers are stored at 2-8°C. shelf life of most of the media is several seeks providing there is no change in the appearance of the medium to suggets contamination or degradation.

Note:-

1. The heat-sensitive ingredients.such as blood or serum, should be brought to room temperature and added when the medium has cooled to about 50°C .
2. A fluid medium should be tested for accurate pH by using a narrow range pH paper.
3. For sterilizing dehydrated culture media. It is necessary to use manufactur‟s instructions.

Ready media:- - Several manufacturers [re[are numerous culture media and market them as „ready prepared media or ready poured plates or as dehydrated powders that have to be reconstituted in the laboratory although the later are not equal in quality to freshly made media, they are significantly labour saving being easy to reconstitute for use. Microbiologists who use ready made media must be constantly vigilant rejecting media, whose performance falls below standards As a precaution a small batch of the supplied medium should be tested before it is taken into use, noting colony size and germination rate as welll as the ability of the medium to perform andy special function. More stringent quality control tests are advisable.

Dehydrated media tend to deteriorate during storage. Storage conditions should comply with the manufactuer‟s instructions or, if unspecified should be cool and dry. There must be adequate mixing and solubilizatin of dehydrated ingredients.

Nutrient broth- A broth base from which nutrient broth, cooked meat broth, etc can be made.

Nutrient agar- Used to make blood agar, heated blood agar,etc

Mac Conkey– A medium without added sodium chloride is chosen as this inhibits the spreading of proteus spp. Sodium chloride can be added, if especially desired.

Sensitivity test agar- An ordinary nutrient agar is not suitable for antibiotic sensitivity testing various inhibitors may be present.specially formuloated media for performing antibiotic sensitivity tests are commercially available, e.g mueller hinton agar.

The manufacturer‟s instructions should always be followed but a few additional general points include:

1. Clean glassware that has been rinsed free from detergents and other chemicals should be used.
2. The appropriate amount of dehydrated medium be weighted out, placed in a flask and distilled water added to it. Glass distilled water must be used, because this is free from chloride and heavy metal ions that can be inhibitory to bacteria.
3. Media not containing agar can usually be dissolved with gentle agitation, but dehydrated media containing agar is best dissolved by bringing to the boil with continuous stirring, using a glass rod or a hot plate that incorporates a magnetic stirrer system.

Handing Agar plates

Do not remove the lid unnecessarily or for prolonged periods of time. Do not lay the lid down on the counter or put the bottom of the petri dish into inverted lid. While inoculatin the agar plate, you may either:

Set the covered plate upside down on the counter. When you are ready to inoculate it with the loop, lift the bottom half (with the media in it) and hold it up vertically for a moment while streaking it. Replace it into the lid while re-flaming the loop. Lift bottom again to continue streaking, etc.

OR

Set the plate right side up on the counter. Lift the lid slightly ajar and hold it at an angle, while you are streaking the place. While this prevents contaminated dust from falling on plate, it may be difficult to see what you are doing.

Note: - Method No - 1 is recommended for examining a plate, which has been incubated in an inverted position. Otherwise, water may condense on the lid and drip down onto the medium, causing the colonies to coalesce.

Handing tubes of broth or agar media

Never lay tubes down on the counter always stand tubes in a rack. If you are right-handed, pick the tube up with left hand, and remove the plug or cap with the little finger of right hand, leaving the thumb and other fingers free to hold the inoculating loop or pipette. Do NOT lay the plug down, or touch anything with it. Holding the tube at about a 45° angle, pass the open end of the tube through the Bunsen burner flame, remove the growth required with the loop or pipette, flame the lip of the tube again, and plug which you are still holding in the crook of the little finger of right hand.

Dispose of all old cultures in the proper containers. Agar plates should not be left in the incubator for more than two days, or they will dry up. When you must save them for a few days, store them in the cold room (see lab coordinator). Do not leave old cultures lying about the room.

Storage of media

Prepared sterilized media in individual screw-capped bottles (e.g., broths and nutrients agars) can be stored at room temperature for weeks, but some deterioration is likely to occur. Poured plates of agar media held on the bench deteriorate quickly and are often contaminated; plates of agar media can be held for short periods not exceeding 7-10 days.

It is essential to have some from of cold storage in the laboratory for the preservation of blood serum and culture media. For a smaller laboratory, one of the domestic refrigerators of 1-2 m^3 capacity is suitable; large laboratories require a correspondingly larger cabinet or an insulated cold room with the refrigerating plant outside. The temperature should be maintained between 4-5°C (39-41°F); it should never be so low as to cause freezing, as this may be deter mental.

Ex -4.2 RECIPIES FOR CULTURE MEDIA

Meat extract

Meat extract contains water-soluble substances of animals tissues, which include carbohydrates, organic nitrogenous compounds, water-soluble vitamins and salts.

Procedure

1. Add 1000 ml of tap water in 500g of fat- free minced meat and place for
2. 24h in the cold room for cold extraction.
3. Strain the mixture through muslin cloth and discard the residue. This will leave the filtrate bright red in color. Remove the surface fat by skimming with filter paper.
4. Boil the filtrate for 15 min. This will coagulate insoluble meat proteins.

5. Filter the fluid through cheesecloth and make up the original volume by the addition of distilled water. This will leave the extract clear and yellow in color.
6. Sterilize at 15 lbs for 30 min.

Nutrient Broth

As the meat extract is deficient in nitrogenous materials, the heat-resistant protein derivatives in the form of peptone are added. Peptone is the principle source of nitrogen it may also contain some vitamins and sometimes carbgohydrates depending upon the king of protein us material digested. Sodium chloride is added to increase the salt content.

Composition

Peptone	1.0g
Sodium chloride	0.5g
Meat extracts	100.ml

Procedure

1. Mix all the ingredients and heat till they are dissolved. Filter the extract though filter paper.
2. Adjust the pH to 7.4 by 0.1 N NaOH solution.
3. Pour the medium in sterile test tubes (1/3 full), flasks, etc, plug with sterile non-absorbent cotton.
4. Sterilize in autoclave at 15 lbs. pressure for 15 min.

Nutrient agar

Nutrient agar is nutrient broth solidified by the addition of agar. It is frequently referred to as „agar" the context making clear that the agar-both mixture is meant and not the pure, non nutrient agar itself, Japanese agar yields a gel of suitable firmness at a concentration of about

2% and New Zealand agar at about 1.5%

Composition

Agar powder	2.0g
Nutrient broth	100.0ml

Procedure

1. Add agar to the nutrient broth.
2. Boil the mixture in running steam for about 30 min to dissolve the agar particles. (if the medium is not clear, add albumin from 2 eggs dissolved in 50 ml water per liter of melted nutrient agar after cooling it to 55°C .The mixture

is then heated for about an hour to coagulate egg albumin with which extraneous material will settle down.)

3. Filter through glass wool and adjust the pH to 7.4
4. Distribute the medium in test tubes (for making slants and butts), bottles of flaks, and autoclave at 15 lbs; pressure for 15 min.

Blood agar

It is a general purpose, enriched and differential solid medium, which supports the growth of most ordinary bacteria. It is also useful to detect and differentiate hemolytic bacteria, S*treptococcus* Blood supplies a number of required substances for the growth of fastidious organisms.

Sterile defibrinated blood at the rate of 5-10% (v/v) is added to the cooled agar base and mixed well before the plates are poured. If bubbles from on the surface of the poured plates, a low Bunsen flame is quickly passed across the surface of the agar before the agar sets. It the sterile blood has been stored in the refrigerator, it should be warmed to 37°C before being added to the agar medium to avoid thermal shock to the red cells.

Collecting sterile blood:

Bovine or ovine blood is most suitable for veterinary bacteriology Sterile blood can be obtained commercially or it can be collected from a youn animal that has no evidence of antibodies to the major veterinary pathogens and has not been treated with antibacterial agents A strict aseptic technique must be used. The area over the jugular vein is clipped and shaved , swabbed with 70 % ethyl alcohol, wiped and allowed to dry thoroughly before venepuncture. To prevent the blood from clotting one of the following methods may be used:

- Collection into a purchased human blood donor kit.
- Collection into a pre-sterilized apparatus consisting of conical flask containing sterile glass beads (3 mm). The flask is agitated continuously during collection and for at least 5 min after obtaining the blood. The defibrinated blood can be decanted into sterile bottles for storage in a refrigerator. The glass beads can be recovered from the fibrin clot and reused.
- A sterile anticoagulant solution, such as 0.2% sodium citrate can be used either in the flask. In the place of the glass beads, or blood can be collected in a sterile syringe and immediately add to the anticoagulant.

Composition	
Nutrient agar	500 ml
Sterile defibrinated blood	25 ml

Procedure

1. Place sterilized agar to 50°C water bath.
2. When it is cooled to 50°C add aseptically sterile defibrinated sheep, oxen or horse blood.
3. pH of the medium should be adjusted to 7.3
4. mix gently and dispense aseptically in sterile patri dishes.

Note:

1. Blood should be free haemolysins
2. Goat or rabbit blood also can be used for most of the pathogens.
3. Use of human blood should be avoided, since certain substances in human blood may be inhibitory to the growth of certain pathogens such as haemolytic streptococci.

Interpretaion

The haemolytic patterns can be interpreted as follows

Alpha haemolysis	Green colour around the colony (*Streptococcus pneumoniae*)
Beta haemolysis	A clear zone around the colony (*Streptococcus pyogenes*)
Gamma haemolysis	No haemolysis(*Streptococcus faecalls*)

Chocolate (heated blood)agar

It is used to grow haemophilus influenzae and other pathogens, which require highly nutritious medium, such as neisseria meningitides and streptococcus pneumonia.

Compostion and procedure

1. It is a prepared by using same procedure used for the preparation of blood agar except that after adding blood, the medium is heated at 70°C in a water bath until it becomes brown in colour. The medium should be mixed gently for about 10-15 min.
2. Allow it to cool to about 45°C.
3. Dispense aseptically in sterilized petri dishes.

Note:

1. The medium should not be overheated.
2. The duration of aseptically in sterilized petri dishes.

Peptone water

The medium is used chiefly as the basal media for carbohydrate fermentation reactions. Nutrient broth may contain a small amount of sugar derived from meat and it is essential that the basal medium to which various carbohydrates are added for fermentation tests should be free from natural sugars. Peptone water is also used to test the formation of indole.

Composition:

Peptone	10 g
Sodium chloride	5 g
Water	1000 ml

Procedure

Dissolve the ingredients in warm water, adjust the pH to 7.4-7.5 and filter. Distribute as required and autoclave at 15 lbs. for 15 min.

MacConkey agar:

This is a differential medium an aid in the identification of Enterobacteriaceae organisms.

Composition

Peptone	2.0g
Sod. Taurocholate	0.5g
Sod. Chloride	0.5g
Lactose	1.0g
Agar powder	2.0g
Neutral red(1% soln)	0.3ml
Water add to	100.0ml

Procedure

1. Dissolve the ingredients with agar powder in the flowing steam.
2. Filter the medium and adjust the pH to 7.6 before dispensing (if the medium is not clear if may be clarified with the help of egg albumin.)
3. Sterilize in autoclave at 15 lbs. pressure for 15 min.
4. Cool to 50-60° C and pour into the plates.

Cooked meat medium (Robertson‟s medium)

This medium is used for the cultivation of anaerobic organisms.

Composition

Fresh ox heart (minced)	500g
N/20 NaOH	500 ml

Procedure

1. Heat the sodium hydroxide solution to boiling.
2. Mix minced meat and simmer for 20 min.
3. Adjust the pH to 7.4 by adding N/20 NaOH solution.
4. Drain off the liquid through a muslin cloth.
5. Dry the minced meat partially on a filter paper.
6. Introduce the minced meat in large sized tubes containing about 10 ml infusion broth so as to occupy about half of the liquid column.
7. Autoclave at 15 lbs pressure for 15 min.

Note:

1. The surface of the medium may be covered with a layer of sterile liquid. Paraffin.
2. The pH of medium should be within the range 7.0-7.4 at room temperature.

Inoculation- Depending on the specimen, cooked meat medium is inoculated using a swab Pasteur pipette, or wire loop. If using a swab this should be inserted to the bottom of the container.

For the culture of strict anaerobes, the medium is best used fresh after being placed (with bottle top loosened) in container of boiling water for 10-15 min to drive off any dissolved oxygen. Allow the medium to cool to room temperature before inoculating it.

Dexoycholate citrate agar(DCA)

It is a selective and differential medium used to isolate salmonella and shigella species. By the addition of extra bile salt this medium can also be used to isolate yersinia species. If can be helpful to differentiate between lactose nonferments and lactose fermenters.

Composition

Beeefextract	5.0 g
Peptone	5.0 g

Lactose	10.0 g
Sodium citrate	8.5 g
Sodium thiosulphte	5.4 g
Ferric citrate	1.0 g
Sodium dexoycholate	5.0 g
Neutral red	0.02 g
Agar	12.0 g
Distilled water	1.01 g

Procedure

1. Dissolve the ingredients in distilled water with great care, do not boil. Cool to 50-55°C and make final volume to 1000 ml.
2. Adjust the pH of the medium to 7.3
3. Mix well and dispense aseptically in sterile petri dishes.

Eosin-Methylene Blue Agar

It is used as selective and differential medium for the growth of members of *Enterobacteriaceae* and lactose fermenting organisms. Other organisms are inhibited by the dye. *Escherichia coli* particularly forms bluish black coloured colonies with metallic sheen. Other coliform organisms form mucoid and brownish colonies.

Composition

Peptone	40.0g
Lactose	5.0 g
Sucrose	5.0g
Dipotassium hydrogen phosphate	2.0g
Agar	17.5g
Eosin (Y)	0.4g
Methylene blue	0.065g
Distilled water	1.01

Procedure

1. Dissolve the ingredients by heating and make final volume to 1000 ml by using distilled water.
2. Autoclave at 15 lbs; for 15 min.
3. Cool to 50-55° C and dispense aseptically in sterilized petri dishes.

Lownstein Jensen acid medium (modified)

Lownstein Jensen (LJ) acid medium is used to isolate mycobacterium tuberculosis and other mycobacteria.

Homogenized whole eggs suspension (6-8 fresh hen"s eggs)	275.0ml
Hydrochloric acid, 1 mol/1 (IN)	8.0ml
Salf glycerol solution[1]	153.0ml
Malachite green, 20g/1 (2% w/v)[2]	2.75ml
Penicillin G (benzyl penicillin)	25,000.0 Ul
Salt-glycerol solution	
Potassium dihydrogen phosphate (KH_2PO_4)	6.3g
Magnesium slphate ($MgSO_47H_2O$)	0.3g
Glycerol	12.0ml
Distilled water	600.ml

Mix the glycerol with the water. Weight the chemicals and dissolve in the glycerol water. Dispense in 153 ml amounts in screw cap bottles. Sterilize by autoclaving at 10 lbs for 15 min.

Store in a cool dark place.

Malachite green solution

Dissolve 1g of malachite green in 50ml of water. Filter and sterilize by autoclaving at 10 lbs for 15 min. Store in a cool dark place.

Procedure

1. Wash the eggs by scrubbing them carefully with soap and water followed by rinsing in clean running water. Immerse the eggs in 70% v/v ethanol for 10-15 min.
2. Using a terile knife, break the eggs into a sterile flask (premarked to hold 275 ml), which contains a few sterile glass beads. Mix well until the egg yolks and whites are completely homogenized.
3. Strain the egg mixture through sterile gauze or muslin into a terile bottle of about 500 ml capacity.
4. Add aseptically the sterile salt glycerol solution, hydrochioric acid, malachite green solution, and penicillin. Mix well for several minutes.
5. Dispense aseptically in 4 ml amounts in sterile screw-cap bottles.
6. Slope the bottles on a tray and inspissate at 80°C for approximately 1h.

Note:

1. The side of medium should end about 10 min away from the neck of the bottle. pH of medium shuld be within the range 6.4-6.8 at room temperature.
2. Store the medium in a cool dark place or at 2-8°C, making sure the bottle tope are tightly screwed.

Selenite F Broth (Modified)

Selenite F (Faeces) both is used as an enrichment medium for the isolation of salmonella species.

Composition

Sodium hydrogen selenite	0.4g
Peptone	0.5g
Mannitol	0.4g
di-sodium hydrogen phosphate anhydrous ($Na_2 HPO_4$)	1.0g
Distilled water	100.0ml

Procedure

1. With case, mix dry ingredients in the water and heat to 75-80°C to dissolve (place the flask in a container of hot water).
2. If the reaction of the medium is not near pH 7.0 adjusts it to the correct pH.
3. Dispense the well-mixed medium in 5 ml amounts in screw-cap universal type bottles to give a depth of 25mm.
4. Sterilize by selective (with caps loosened) for 20 min. tighten the bottles caps after sterilizing. A small amount of red precipitate may form. This will not interfere with the performance of the medium.

Note: Do not steam for lnger than 20mm. Overtheating can damage the medium. Never autoclave selenite broth.

Inoculation:

If the faecal specimen is formed or semi-formed, make a thick suspension of it in about 1 ml of sterile saline and after the faecal debris has sedimented, inoculate the medium with about 0.5 ml of the supernatant fluid. If the specimen is unformed, use several loop full.

Composition

Beef extract	300.0g
Casein hydrolysate	17.5g
Stareh	1.5g
Agar	10.0g
Distilled water	1.0l

Procedure

1. Emulsify the starch in a small amount of cold water.
2. Pour into the beef extract and ad casein hydrolysate and the agar.
3. Dissolve the ingredients by heating gently at 100°C with agitation.
4. Adjust pH of the medium to 7.4.
5. Sterilize in an autoclave at 15 lbs for 15 min.

Sabouraud dextrose agar

It is used to culture yeatsts and moulds
Composition

Peptone (mycological)	10g
Glucose	20g
Agar	15g
Distilled water	1000ml

Procedure

1. Dissolve the ingredients in distilled water by steaming.
2. Adjust pH of medium 5.4-5.8.
3. Sterilize at 10 lbs for 15 min.

Ex 4.3 ADJUSTMENT OF P^H OF NUTRIENT BROTH

Materials: nutrient broth, phenol red indicator discs (standard color) and lovibond comparator set, uniform size test tubes to fit in the holes of comparator set, N/10 and N/10 solutions of NaOH and HCL, pipette 10ml graduated to 1:100 phenol red solution.

Procedure:

1. Place 5 ml of broth medium in one of the test tubes.
2. Add 0.5ml of phenol re indicator to the medium. Mix thoroughly and place of the holes of the comparator set.
3. Add 5ml of broth medium in the second hole of the comparator set to serve control.
4. Fix phenol red disc in the comparator set. Rotate the disc while viewing the same through the window of the instrument until the colour of the disc and medium with the indicator matches.
5. If the colour of the medium with the indicator does not compare with the colour of the desired pH of the disc, add mes\asured quaintly of N/10 NaOH

or N/1 HCL, as the case may be, till it matches with the colour of the desired pH.

6. From the amount of N/10 NaOH or N/10 required to adjust the desired pH of 5ml sample. Calculate the amount of N/1 NaOH or N/1 HCL needed to adjust the pH of the total volumes of the medium as follows:

Results:

Amount of N/10 NaOH or N/10 HCL = Amount of N/10 NaOH or N/10
Required to adjust pH of 100ml required to adjust pH of 5ml x 20

Ex. 4.4 Preparation of Petri Plates of Nutrient agar.

Introduction: An culture plate of standard size (10 cm diameter) can take 20 to 25 ml of sterile culture media. The use of petri dishes is convenient for aerobic culture of an organism. The petri dishes allow the organism to be isolated in pure colonies from the commensals.

Materials: Nutrient agar, autoclave, petri dishes, incubator.

Procedure:

1. Prepare and sterilize themedium by autoclaving in a conical flask at 15 lbs for 15 min.
2. Cool the culture medium to about 55°C.
3. Under aseptic conditions pour the melted culture medium (15 to 20 ml) into sterilized petri dishes.
4. Allow the medium to solidily in a flat position.
5. Koap the petri dishes in aninverted position overnight; in incubator at 37°C.
6. Store the petri dishes in the inverted position in a refrigerator at 2-8°C.

Objective: To prepare slants of Nutrient agar.

Introduction: Agar slants are test tubes containing an agar medium in the form of slopes. The agar slants are prepared by placing the test tubes containing melted agar, during cooling at an angle of 20°C or less. The sloped surface of the agar can easily be inoculated with a nichrome loop or needle, since it provides more surface area.

Materials : Nutrient agar, autoclave, sterile Pyrex test tubes, incubator.

Procedure:

1. Dispense about 5.0ml of molten agar medium in each test tube(15x150mm)
2. Sterilize the tubes by autoclave at 15 lbs for 15 min.
3. Place the test tubes in an inclined position (at about 20°C angle) and allow the melted agar to solidify at room temperature (25°C$\pm$5°C).
4. Store in the refrigerator at 2-8°C.

Ex-5 MEDIA INOCULATION & PURE CALTURE TECHNIQUE

INOCULATION OF MEDIA BY DIFERENT METHODS

In is absolutely essential to deal with pure cultures of bacteria for various studies. In order to obtain and maintain a pure culture, techniques which do not permit the introduction of contamination, must be used. For the manipulation of pure culture, in addition to the use of sterile containers and media, aseptic techniques should be adopted so that no extraneous organism gets into the culture from the air. The most commonly used techniques for the isolation of pure culture is by streaking the inoculums on the solid medium to obtain single isolated colonies. Until the contaminated material is diluted to the extent that the inoculum is likely to contain only one viable organism, it is not possible to obtain pure culture by using liquid medium. On the other hand on solid medium if the inoculum is diluted by successive streaking, single isolated colonies are produced on the track of inoculation. Each colony is likely to developed from a single bacterial cell and is a pure culture. Once pure culture has been obtained, on subsequent culture any contamination colonies are easily detected by their appearance. Each bacterial species maintains its morphological, cultural and physiological characteristics on subculturing. In this exercise the students will learn various techniques of inoculation some of which are also helpful for pure culture isolation.

A. INOCULATION BY STREAK PLATE METHOD

This method is most commonly used for pure culture isolation.

Materials

E.coli culture, inoculation loop and nutrient agar plate.

Procedure

1. Sterilize inoculation loop and pick up a small amount of E. coli culture aseptically near the flame of gas burner.
2. Raise the lid of the Petri dish and make 3 to 4 parallel streaks across the surface of the medium at one side of the dish.
3. Sterilize the inoculation loop on the flame and draw 3 to 4 parallel streaks across the first streaks.
4. Repeat the streaking 2 to 3 times more across the previous streaks spacing the streaking evenly. This ensures dilution effect and facilitates growth of discrete isolated colonies.
5. Incubate the plate at 37°C for 24 to 48 hours.

Results

Examine the plate and make a drawing.

B. INOCULATION BY SPOT METHOD

Material

E.coli culture, inoculation loop and a nutrient agar plate.

Procedure

1. Sterilize inoculation loop and pick up a small amount of E.coli culture aseptically.
2. Raise the lid of the dish slightly and rub the inoculums at a spot on the surface of the medium.
3. Incubate the plate at 37°C for 24 to 48 hours.

Results

Examine the plate and make suitable drawings.

C. INOCULATION BY POUR PLATE METHOD

Mateiral

E.coli broth culture, nutrient broth tube (10ml), nutrient agar tube (15ml), and sterile petri dish.

Procedure

1. Melt nutrient agar in a boiling water bath.
2. Allow nutrient agar to cool at 46°C to 48°C.
3. Add a loopful of broth culture in the nutrient broth tube and mix thoroughly.
4. Transfer and loopful of the diluted culture to melted nutrient agar and rotate the tube rapidly for a few seconds.
5. Pour the content into the sterile petri dish and spread evenly. Avoid creating air bubbles.
6. After the inoculated nutrient agar is solidified, incubate the plate at 37°C for 48 hours. The plate should be incubated upside down.

Result

Examine the place and make suitable drawing

D. INOCULATION BY SHAKE CULTURE METHOD

This method is useful to isolate single colonies of anaerobic organisms by using dilute inoculums.

Materials

E.coli broth culture, nutrient broth tube (10 ml) 1, nutrient agar tube (15 ml) 1.

Procedure

1. Add a loopful of the *E.coil* broth culture to nutrient broth tube and mix thoroughly.
2. Add a loopful of the diluted broth culture in melted nutrient agar tube at 45°C. shake agar tube thoroughly to disperse the organisms uniformly in the medium. Stopper the tube.
3. Keep the inoculated tube in an upright position to solidify the medium. Incubate the tube at 37°C.
4. After 48 hours of incubation first heat the tube at the sides and then at the bottom on the direct flame. The butt of the agar will come out of the tube with a force.
5. Fish out the colonies from the agar butt by cutting the agar with the helop of a knife.

Result

Examine the tube and make a drawing.

E. INOCULATION OF A SLANT (STROKE CULTURE)

Material

E.coli culture, inoculation loop and nutrient agar slant.

Procedure

1. Pick up a small amount of inoculums on the inoculation loop.
2. Insert the loop near the bottom of the slant and draw it gently upwards over the surface of the medium in a zigzag manner.
3. Incubate the slant at 37°C for 24 to 48 hours.

Results

Examine the slant and make a suitable drawing.

F. INOCULATION BY STAB METHOD

Stab inoculation is generally used for gelatin liquefaction.

Materials

E.coli culture gelatin butt and a straight inoculation needle.

Procedure

1. Sterilize inoculation needle and pick up a small amount of *E.coli* culture.
2. With aseptic precautions, remove the cotton plug from the gelatin tube and introduce the inoculation needle perpendicularly into the medium without touching the sides or bottom of the tube. Withdraw the needle through the same path without breaking the medium.
3. Include the tube at 37°C for 24 to 48 hours.

Results

Examine the stab culture and make a suitable drawing.

G. INOCULATION OF LIQUID MEDIUM

Material

E.coli culture, inoculation loop and nutrient broth tube.

Procedure

1. Pick up a small amount of inoculums form the culture plate or tube taking aseptic precautions.
2. Insert the inoculation loop in the broth tube and rub gently by the side of the tube to dislodge the inoculum into the medium.
3. Include the tube at 37°C for 24 to 48 hours.

Results

Record the observations and make a suitable drawing.

EX.6 CULTIVATIN OF BACTERIA ANAEROBICALLY

Aerobic organisms grow in the presence of free oxygen, strict anaerobes in the absence of free oxygen, and facultative anaerobes in the absence as well as presence of free oxygen microaerophilic organisms require reduced oxygen tension for their growth. There are some organisms which require increased CO_2 tension for their growth. Organisms belonging to different gaseous requirements are having different sets of enzymes and hence possess different metabolic pathways. As an example, if anaerobes which do not possess catalase enzyme are grown in presence of free oxygen, they will produce and accumulate H_2O_2 in the culture environment which is injurious for the cells. Organisms possessing catalase on the other hand break H_2O_2 to H_2O and O escape from harmful effect of H_2O_2.

There are usually four methods, which may be adopted for the growth of growth of anaerobic organisms.

A. EXCLUSION OF AIR

The example of this is inoculation by shake culture method.

B. ADDITION OF ANIMAL TISSUES INTO THE MEDIUM

The presence of live or dead tissues (e.g., meat, heart, brain, liver, etc.) in the medium increases is reducing potential. One of the examples of such media is Robertson‟s medium. At the time of inoculation, the medium should be heated to expel the air and cooled before inoculation the medium can also be sealed with sterile liquid paraffin or Vaseline.

C. INCORPORATIO OF REDUCING COMPOUNDS INTO THE MEDIUM

The addition of compounds like glucose, cysteine and thioglycollate in the medium producer a reducing potential which is favourable for the growth of anaerobic organisms.

D. DISPLACEMENT OF OXYGEN FROM CULTUREAL ENVIRONMENT BY INERT GASES LIKE HYDROGEN OR NITROGEN.

This is accomplished in the McIntosh and Fielde‟s anaerobic jar. Hydrogen gas is supplied from the cylinder or produced in the Kipp‟s apparatus by the use of zinc granules and sulphric acid;

$$Zn+H_2SO_4 \rightarrow ZnSO_4+H_2$$

For the organisms requiring additional CO_2 gas may be prepared by the use of marble chips and dhydrochloric acid:

$$CaCO_3+2HCL \rightarrow CaCL_2+H_2O+CO_2$$

Materials

Cultures of *Cl. Chauvoe* and B. *subtilis,* MaIntosh and Fielde‟s jar. Vacuum and pressure pump, supply of hydrogen gas from cylinder or Kipp‟s apparatus, blood agar plates 4, Robertson‟s medium tubes 2, thioglycollate broth tubes2, methylene blue indicator tube consisting of equal volume of:

(a) NaOH soln. (N/10), 6 ml in 94ml of water.
(b) Aqueous soln. of methylene blue (0.5%), 3 ml in 97ml of water.
(c) Glucose 6gm in 100 ml of water with a small crystal of thymol.

Procedure

1. Inoculate 2 blood agar plates and one tube each of Robertson‟s and thioglycollate media with *cl.chauvoei* and another similar set of media with *B. subtilis.*
2. Place 2 blood agar plates one inoculated with *cl.chauvoei* and the other with *B. subtilis* in the McIntosh and Field‟s jar. Before covering the lid of the jar, place a tube of methylene blue indicator after heating and proceed as follows:
   (a) Connect anaerobic jar with a vacuum pump. Allow the pump to operate till the air is completely exhausted from the jar and mercury manometer reading reaches to 710mm.
   (b) Fill the jar with hydrogen gas. The manometer reading will now show 760 mm, i.e. normal atmospheric pressure.
   (c) Repeat steps (a) and (b). This will completely exhaust oxygen gas from the anaerobic jar and the same will be replaced by hydrogen.
   (d) Pass weak electric current through the two electric terminals provided on the lid of the jar to remove remaining traces of oxygen, if may. The current passes through the palladiumized wire which acts as a catalytic agent for the removal of the oxygen from the jar. When there is complete anaerobic condition in the jar, the indicator will remain colourless except slight green colouration on the top.
   (e) Disconnect the anaerobic jar from the vacuum pump, Kipp‟s apparatus or H_2 gas cylinder andelectric main.
3. Include the inoculated plates in the jar and other culture media in the incubator at 37°C for 48 hours.

Results

Compare the culture media to determine the presence or absence of growth. Record the results as +++, ++, + or 0.

Ex -7- MORPHOLOGY & CULTURAL CHARACTERISTICS OF BACTERIA

MORPHOLOGY OF BACTERIA

Bacteria are morphologically different. This characteristic is helpful in the identification of bacteria. Routine study of bacterial morphology should include examination of stained preparations. Motility should be examined by hanging drop preparation. According to the requirement, certain cell structures may be stained. Stained preparations should be made preferably from fresh agar slant cultures. Smears of *Streptococcus* should be made from a liquid culture. Morphology of bacteria may be described under the following terms.

1. Shape. Spherical, coccobacillary, short or long rods, filamentous, comma-shaped, curved or spiral forms,
2. Size. Spherical are measured in diameter and rod-shaped in length and breadth. Measurement is expressed in microns (μ).
3. Sides. Straight, concave, bulging parallel or irregular.
4. Ends. Round, pointed, truncate or concave.
5. Axis. Straight or curved.
6. Arrangement. Singly, in twos, in groups of four or eight, in grape-like bunches, in long chains, or scattered irregularly, may be in bundles, angular arrangements or in Chinese letters.
7. Pleomorphic bacteria may differ from their typical shape. Some of the older cultures may depart greatly from their typical forms, and may show spindles, clubs, and filaments, swollen and branching forms. Variation may be both in shape and size.
8. Capsule. May or not be present. When present, size should be noted. This requires special staining.
9. Flagella. Bacteria may be atrichous, monotrichous, amphitrichous, lophotrichous or peritrichous.
10. Motility. Present or absent. This should be tested in young broth culture. Motility should not be confused with Brownian or molecular movement which is a to and fro motion without change in position.
11. Spores. Some species are sporulated. Spore may be spherical or oval. The location of the spore may be central, subterminal or terminal in the cell and may or may not bulge the size of the bacteria mature spores do not stain with Gram‟s Method.
12. Staining reaction. Reaction to Gram‟s or Ziehl-Neelsen‟s stain should be mentioned. The staining may be uniform, unipolar, bipolar, beaded or there may be presence or absence of metachromatic granules. Some cells may take deeper colour than others.

Materials

Nutrient agar slant cultures of *staph. Aureus, C. ovis, E.coli* and B. subtilis, glass slides and Gram‟s stain.

Procedure

1. Make smears of different microorganisms, air dry and fix on the lens.
2. Stain the smears with Gram‟s stain.
3. Examine smears for bacterial morphology under oil immersion lens.
4. Test the cultures for motility by hanging drop preparation.

Results

Select suitable field of each bacterial species under microscope, make sketch and record morphology.

Ex -8 - BACTERIAL STAINS & STAINING

Ex .8.1 – NEGATIVE STAINING

In this method of staining the bacteria only their background is stained. The organisms are seen as clear transparent objects in a stained background. In this method of staining there is little chance of distortion of the bacterial morphology. The commonly used stains arenegrosin and India ink. In this exercise nigrosin solution will be used.

Composition

Water	10gm
Distilled water	100ml

Immerse the stain in boiling water for 30 minutes and then add 0.5 ml formalin. Filter twice through filter paper.

Materials

Nigrosin solution, *B. subtilis* culture.

Procedure

1. mix 1 loopful of the culture with equal amount of stain on the slide.
2. Allow the mixture to dry in the air and examine under microscope with oil immersion objective.

Results

Select a suitable field under the microscope and make drawing
Mentioning the magnification.

Ex -8.2 SIMPLE STAINING

Most bacteria can readily be stained with solution of basic dyes. In this exercise aqueous methylene blue Loeffer‟s alkaline methylene blue and dilute carbol fuchsin stains will be used to stain bacteria.

A. AQUEOUS METHYLENE BLUE

This is a simple bacterial stain for routine use. This can also be used as a counter stain with Ziehl-Neelsen‟s method of staining for acid fast organisms.

Composition

Sturated solution of methylene blue (approx. 1.5%)	5ml
Distilled water	95ml

The ingredients are combined and filtered.

Material

Staph. Aureus and *E.coli* culture plates, and aqueous methylene blue stain.

Procedure

1. Prepare culture smears of each organism as follows:
   (a) Place a small amount of water on the slide with the help of a inoculation loop.
   (b) Flame the loop, raise the lid of the petri dish near the flame and remove a portion of colony on the tip of the loop.
   (c) Emulsify the growth in the drop of water on the slide in a circular motion and spread it in about 1½ sq. cm diameter. Flame the needle again and close the lid of the plate.
   (d) Air dry the smear and fix it over the flame by passing the slide 2to3 times with smear upwards:
2. Pour the stain and allow acting for about ½ to 1 minute.
3. Watch with water, blot and dry before examination.

B. LOEFFLER'S ALKALINE METHYLENE BLUE

This stain is particularly useful to show McFadyean"s reaction with *Bacillus anthracis.* This is also used to demonstrate metachromatic granules in case of *Corynebacterium* and as a counter stain with Ziehl-Neelsen"s stain for acid fast organisms.

Composition

Methylene blue saturated alcoholic soln.	30ml	
Pot. Hydroxide (1% aqueous soln.)		1 ml
Distilled water		99ml

Add KOH in water and combine with Methylene blue solution before filtration.

Materials

Staph. aureus and *E. coli* culture plates and staining solution.

Procedure

1. Prepare bacterial smears and fix on the flame.
2. Pour stain on the smears and allow to act for 4 to 5 minutes
3. Wash with water, blot and dry before examination.

C. DILUTE CARBOL FUCHSIN

This may be used as a counter stain with Gram‟s method of staining and is not much used as a simple stain.

Composition

Concentrated solution

Basic fuchsin	1gm
Absolute alcohol	10ml
Phenol 5% aqueous soln.	100ml

Dissolve the dye in alcohol and add phenol solution. This is used for staining acid fast bacilli. For simple staining or as a counter stain with Gram‟s stain. Concentrated solution is diluted as follows:

Concentrated solution	100 ml
Distilled water	1000 ml

Materials

Culture of *Staph. aureus* and *E. coli* and dilute carbol fuchsin stain.

Procedure

1. Prepare smears and fix over flame..
2. Apply stain and allow to act for about 20 seconds.
3. Wash with water, blot and air dry before examination.

Results

Select suitable field under the microscope, make drawing and describe staining characteristics.

Ex -8.6 – DIRRERENTIAL STAINING

These stains show differences among different types of bacteria and therefore are useful their identification. The staining procedure involves more than one dye. Gram‟s and acid fast stains belong to this category.

A. GRAM'S STAIN

This is most useful and frequently employed stain in bacteriology. Bacteria stained by Gram‟s method fall into Gram positive and Gram negative groups. Gram positive bacteria

retain the crystal violet colour and hence appear deep violet or blue in colour. Gram negative bacteria lose crystal violet colour when treated with a decolourizer and are counter stained by safranin and here appear red in colour. There are many bacteria which are Gram variable rather the true Gram positive or Gram negative. The old cultures of some Gram positive organisms are easily decolourized and therefore take the red colour of counter stain. The Gram positive and Gram negative bacteria differ greatly. The differences are shown in table

Composition

A. Ammonium oxalate crystal violet (Hucker‟s)

Solution 1

Crystal violet	2 gm
Ethyle alcohol (95%)	20 ml

Solution 2

Ammonium oxalate	0.8 gm
Distilled water	80 ml

Mix solutions 1 and 2 and filter.

B. Lugol‟s iodine solution

Iodine	1 gm
Pot. Iodide	2 gm
Distilled water	300 ml

Dissolve the ingredients and filter.

C. Ethyl alcohol (decolourizer)

The difference between Gram positive and Gram negative bacteria

Property	Gram positive	Gram negative
Cell wall thickness	Greater	Less
Amino acids in cell wall	Few kinds	Numerous kinds
Hexosamine content of cell wall	More	Less
Magnesium content	greater	Less

Digestion of killed organisms by trypsin or pepsin	Resistant	Not resistant
Resistance to strong alkai (1%KOH)	Not dissolved	Dissolved
Apparent isoelectric point by staining	pH 2-3	Abut 4-5
Susceptibility to acriflavin dye	Marked	Less marked
Inhibition by sodium azide	Resistant	Less resistant
Permeability to dye in living state	More permeable	Less permeable
Bacteriostatic action of iodine	More susceptible	Less susceptible
Nature of foxins produced	Exotoxins	Endotoxins and antigens of boivin type
Nutrient requirements	Generally complex(none autotrophic	Relatively simpler (many species autotrophic)
Susceptibility to penicillin	Greater	Less
Susceptibility to anionic detergents	Very susceptible	Much less (susceptible only in acid media)
Bacteriostatic action of tellurites	More resistant	More sensitive

Materials

Staph. aureus and *E. coli* culture plates, staining solutions and reagents A,B,C, and D.

Procedure

1. Prepare bacterial smears and fix over flame.
2. Pour ammonium oxalate crystal violet stain and allow to act for 1 min.
3. Wash with tap water.
4. Add Lugol‟s iodine to act for 1 min.
5. Wash with tap water and blot dry.
6. Decolorize with 95% ethyl alcohol for about 30 seconds gently agitating the slide till no colour comes out from the smear. Blot dry.
7. Counter stain for about 10 to 20 seconds with safranin solution.
8. Wash with tap water, dry and examine.

Interpretation

Gram positive organisms will be deep violet and Gram negative red.

Results

Select suitable field under the microscope, make drawings and describe staining characteristics

B. ZIEHL- NEELSEN'S STAIN

Species belonging to the genus *Mycobacterium* do not stain readily by simple staining procedures. Their staining is facilitated by heat. Once stained they retain the colour of the dye even when treated with a suitable decolourizer. These organisms are designated as acid fast the organisms which are decolourized, take counter stain and are non-acid fast.

Composition

A. Concentrated carbol function.

B. Acid alcohol (decolourizer)

Ethyl alcohol (95 %)	97 ml
Concentrated HCI	3 ml

C. Loeffer‟s alkaline methylene blue

Materials

A portion of the formalin preserved tuberculous lung, and staining solution A, B, and C.

Procedure

1. Cut a piece of the tuberculous lung, scratch the cut surface with a knife edge and spread evenly on the slide. Dry the smear and fix over the flame.
2. Flood the smear with carbol fuchsin and steam for 3 to 5 min. Do not boil or char the stain stain should not dry on the slide.
3. Wash with water.
4. Decolourize with acid alcohol until the preparation is faint pink or colourless (about 15 to 20 seconds).
5. Wash with water
6. Counter stain with Loeffer‟s methylene blue for about 30 seconds.
7. Wash with water, blot carefully and dry before examination.

Interpreation

Acid fast bacteria will stain red and non-acid fast and tissue debris blue.

Results

Select typical field under the microscope, make drawing and describe staining characteristics.

Ex -8.4 Spore Staining

Objective: To stain bacterial endospores.

Materials : Sporulating culture of B.*subtilis,* staining reagents normal saline solution (NSS),nichrome loop, glass slides, sompound microscope, and fire torch.

(A) Modified zeihl Nelson's method

Reagents:

1. Concentrate carbol fuchsin solution (Ex 8.2)
2. Sulphuric and solution (0.5% v/v)
3. Loeffer"s alkaline methylene blue (Ex8.2)

Procedure

1. Prepare a thin smear of the bacterial culture on a clean glass slide in NSS, dry in air, and fix over a flame.
2. Pour conc. Carbol fuchsin over the smear and steam for 3 to 4 min by placing the fire torch beneath the slide.
3. Wash with tap water.
4. Decolourise with 0.5% sulphuric acid.
5. Wash with tap water.
6. Counter stain with loeffer"s blue for 2 min.
7. Wash the tap water, blot dry, and observe under the oil immersion objective of compound microscope.
8.

Interpretaion: The spores will stain red and vegetative cells blue.

Result:

B. Schuffer & Fultion's method

Reagents :

1. Malachite green solution (5% w/v).
2. Safranin solution (0.5%).

Procedure

1. Prepare a thin smear of the bacterial culture on a clean glass slide in NSS dry in air, and fix over a flame
2. Place a piece of blotting paper over the smear and saturate with malachite green.
3. Let the malachite green sit on the slide for one minute and proceed to the next step.
4. Holding the slide with forceps, carefully heat the slide in the flame of a Bunsen burner until the stain just begins to steam. Remove the slide from the heat until steaming stops; then gently reheat. Continue steaming the smear in this manner for five minutes As the malachite green evaporates, continually add more. Do not let the paper dry out.
5. After five minutes of steaming, wash the excess stain and blotting paper off the slide with water. Don"t forget to wash of any dye that got onto the bottom of the slide
6. Blot the slide dry.
7. Now flood the smear with safranin and stain for one minute
8. Wsh off the excess safranin with water, blot dry, and observe using oil immersion microscopy.

Interpretation: The spores will stain green and vegetative cells pink.

Ex – 8.5 STAINING METACHROMATIC GRANULES

Objective : To stain metachromatic granules.

Materials : Culture of corynebacterium diptheriae, staining reagents, nichrome loop, normal saline solution (NSS) nichrome loop, glass slides, and compound microscope.

Albert‟s staining method

Reagents :

Toluidine blue	0.15 g
Malachite green	0.20 g
Glacial acetic acid	1.00 ml
Alcohol (95%)	2.00 ml
Distilled water	100.00 ml

Grind and dissolve the dyes in alcohol, add water and then add acetic acid. Let the mixture stand for 24 hours and then filter. Label the bottle, and store in the dark at room temperature. The stain is stable for several months.

Albert stain

Lodine	2.0g
Potassium iodide	3.0g
Distilled water	300.0ml

Dissolve iodine and potassium iodide in water by grinding in a mortar with a pestle. Filter through a filter paper. Label the bottle, and store in a dark place at room temperature.

Procedure

1. Prepare a thin smear of the bacterial culture on a clean glass slide in NSS, dry in air, and fix the dried smear using alcohol.
2. Cover the smear with Albert stain I. let it stands for two minutes.
3. Wash off the stain with clean water. If the tap water is not clean, use filtered water or clean boiled water.
4. Cover the smear with Albert stain II. Let is stand for two minutes. Wash with water.
5. Wipe the back of the slide clean, and place in a drining rack for the smear to air-dry
6. Examine the smear microscopically, first with the 40x objectives to check the staining and to see the distribution of material and then with the oil immersion lens to look for bacteria containing metachromatic granules.

Interpretation: The metachromatic granules will stain green blue and cells take green stain.

Ex -8.6 Capsule Staining

A. Negative Method

Objective: to demonstrate capsule in a given bacterial culture.

Materials: culture of *Klebsiella* spp. Staining reagents nichrome loop, glass slides, and compound microscope.

Negative staining

Principal: In the method instead of staining the bacteria, only their background is stained. The organisms appear as clear transparent objects against a stained background. There is little change of distortion of the bacterial morphology in such method of staining. The commonly used stains are nigrosin and india ink.

Interpreation: The organisms appear as colourless (refractive) objects against a bluish-black background.

Results:-

B. Hiss Method

Principle: In this method crystal violet is applied as a stain. On application of 20% $CuSo_4$ solution, an osmotic difference is created due to which the stain diffuses towards the outer surface of the cell. This results into appearance of capsule as a light violet coloured layer (halo) against deep violet coloured bacteria cell.

Reagents

1. Copper sulphate solution (20% w/v).
2. Crystal violet solution (1% w/v).
3. Blood serum
4. Methanol or absolute alcohol (70% v/v).

Procedure

1. Transfer aseptically a loopful of serum on a clean and dry slide.
2. Mix it with loopful of culture.
3. Spread the suspension to prepare a thin smear and dry in air.
4. Fix fixing cover the smear with one or two drops of 70% v/v methanol or alcohol. Leave the alcohol on the smear for a minimum of 2 min or until the alcohol dries in the smear.

5. Cover the smear with crystal violet stain, and heat gently until the steam just begins to rise. Lvave the stain for one minute.
6. Wash off the stain with the copper sulphate solution.
7. Wipe the back of the slide clean, and place in a draining rack for the smear to air-dry.
8. Examine the smear microscopically, first with the 40x objectives to see the distribution of material and then with the 100 x oil immersion objectives to look for capsulated bacteria.

Interpreation : The bacterial cell stains dark purple and capsule pale blue (mauve) colour

Result : Draw the diagram of observed field under microscope

Ex -9 BIOCHEMICAL CHARACTERIZATIONS OF BACTERIA

Bacterial species different in their capacity to utilize different carbohydrates, proteins and lipids. This is taken advantage of in identification and taxonomy of bacteria; the process being sometimes also known as „biotyping".

The types of biochemical reactions each organism, undergoes act as a "thumbprint" for its identification. This is based on the following chain of logic.

- Each different species of bacterium has a different molecule of DNA (i.e DNA with a unique series of nucleotide bases).
- Since DNA codes for protein synthesis then different species of bacteria must, by way of their unique DNA, be able to synthesize different protein enzymes.
- Enzymes catalyze all the various chemical reactions of which the organism is capable. This in turn means that different species of bacteria must carry out different and unique sets of biochemical reactions.

Some of the commonly used biochemical tests along with their main use and principle are presented in the table using either or combinations of the following one can carry out these biochemical tests:

1. Conventional biochemical tests usually carried out singly in small bottles or tubes.
2. Commercial media prepared in tubes, incorporating several biochemical tests, e.g, TSI medium.
3. Miniatureised methods that are available commercially and use small amounts of media in small chambers, e.g API 20 *E. coli* kit.
4. Automated microbiology system.

Preparation of inoculum for test media

The validity of the identification of an anknown bacterial culture by its reactions in a range of biochemical tests depends absolutely on the use of a pure culture of the bacterium for inoculation of the test media. It is recommended, therefore that the chosen colony should first be plated out on a non-selective culture medium and that an isolated colony on the secondary plate should be used as the inoculum for the tests.

If a larger number of test media have to be inoculated then a single colony should first be sub cultured on an agar slope, on in a tube of broth, and this subculture should be used to inoculate the test media. The subsulture can then be preserved for making confirmatory or other different tests on subsequent days. It is an unsound practice to use another, apparently similar colony from the primary plate to provide inocula for additional tests.

Control for tests

The sterility of each batch of test medium should be confirmed by incubating one or two inoculated tubes of the batch along with the inoculated tests. In the uninoculated

tubes show the evidence of bacterial growth, the tests and the remainders of that batch of medium should be discarded.

Control test are also done to confirm that the test media have been made up correctly and that they are used and observed under the proper conditions. One tube of each batch of test medium is inoculated with a stock culture of a bacterium known to give a positive reaction negative controls are incubated and examined along with the tests.

Test	Main use	Principle	Positive test
Catalase	To differentiate *staphylococci* from *streptococci*	The enzyme catalase breaks down hydrogen peroxide to oxygen and water.	Release of oxygen bubbles
Citrate	Todifferentiate *enterobacteria* from other bacteria	Organism uses citrate as its only source of carbon, producing an alkaline reaction with a colour change of indicator	Blue and turbid medium
Hydrogen sulphide (H2S)	To differentiate *enterobacter; Bacterioides* spp. And *brucella* spp.	Sulphur-containing amino acids are decomposed with the release of H_2S which is detected by an iron salt	Blackening in medium
	To differentiate Gram-negativerods, especially *E.coli*	Tryptophan is broken dwon with the release of indole which reacts with dimethyl amino ben-zaldhyde.	Reddening of strip or medium
Litmus milk decolourisation	To identify enterococci and some clostridia.	Litmus milk is reduced with a decolourisation of the litmus.	Medium becomes white or cream coloured
	Todifferentiate M*ycobacterium* spp. And Gram negative bacteria	The enzyme nitrate r	Reddening of medium
	To help identity *Vibrio,Neisseria, Pasteurella, Pseudomonas supp.*	Oxidse enzyme oxidize phenylenediamine.	Deep purple colour on reagent paper

Oxidation fermentation	To help identify P.asruginosa and differentiate other bacteria	Aerobic (oxidative) and anaerobic (fermentative) utilization of carbohydrate	Oxidative : yellow in open tube, green in closed tube. Fermentative: yellow in both tubes
Phenylalanine deaminase	To differentiate Proteus and providencia from other Enterobacteria.	Phenylalanine is broken down with the production of phenylpyruvic acid. The acid is detected by iron ferric chloride	Slope becomes green
Tween hydrolysis	To identity slow growing Mycobacterium spp.	The enzymes lipase hydrolyses Tween 80, producing oleic acid which changes the colour of the indicator	Pink red medium
	To help identify Proteus.	The enzymes lipase hydrolyses urea,producing ammonic which changes the colour of the indicator	Red pink medium
Voges-proskauer	To differentiate Enterobacteria	Organism ferments glucose with acetoin production. Acetion is oxidized to diacetyl which reacts with creatine	Pink colour developing slowly in medium
	To differentiate Enterobacteria	Organism ferments g acidity in a buffere change of indicator.	Bright red medium

Catalase test

Principle: Catalase is the name of an enzyme found in most bacteria, which initiates the breakdown of hydrogen peroxide (H_2O_2) into water (H_2O) and free oxygen (O_2) Most bacteria are catalase-positive; however certain genera that don"t carry out aerobic respiration, such as *Streptococcus, lactobacillus,* and *Clostridium,* are catalase-negative.

Requirements: cultures of *Staphylococcus* aureus and *Streptococcus* lactis, 3% hydrogen peroxide.

Procedure: Add a few drops of 3% hydrogen peroxide to each culture and look for the release of exyen as a result of hydrogen peroxide breakdown alternatively. Take few

drops of 3% hydrogen peroxide in a test tube and transfer bacterial colonies using a glass rod or a plastic loop.

Note; Nichrome wire loop should not be used as it may give a flase positive reaction. The test should be performed on a blood- free medium.

Interpretation: if the organism has catalase activity, foaming or effervescence will appear within 10-30 seconds

Oxidase test

Principal: A positive oxidase reaction reflects the ability of a microorganism to oxidize certain aromatic amines, such as teramethyl -p- phenylenediamine (TPD) producing colored end products. This is due to the activity of cytochrome oxidase in the presence of atmospheric oxygen. One use of the test is for the preliminary identification of neisseria and moraxella species, which are both oxidase positive gram-negative diplococcic. Members of Enterobacteriaceae are oxidase negative.

Requirements: cultures of Staphylococcus aureus and Neisseria, 1% aqueous soln. tetramethyl-p-phynylenediamine dihydrochloride, wooden stick.

Procedure: using a sterile wooden stick, remove 2-3 colonies from each culture to be tested and smear on a piece of filter paper. Add a drop of the spot test (TPD) reagent to each spot.

Interpreation: If the organism has oxidase activity it will turn purple within 10-30 seconds.

Results

Oxidase test (+or-)	Bacteria species	
	S. aureus	*Neisseria*

Tests for Metabolism of Carbohydrates and Related Compounds

Bacteria differ widely in their ability to metabolise carbohydrates and realted compounds. For purpose of identification these differences can be demonstrated by four varieties of tests:

1. Tests of distinguish between aerobic and anaerobic breakdown of a carbohydrate.
2. Tests to show the range of carbohydrates and related compounds that can be attacked.
3. Tests for specific breakdown products.
4. Test to show ability to utilize a particular substrate.

Oxidation- Fermentation (O/F) test (hugh and leifson's method)

This test is used to determine the oxidative or fermentative metabolism of a carbohydrate by the bacterium. The medium is semi-solid and usually contains glucose as the test sugar and bromothymol blue as the pH indicator.

This test is used to differentiate those organisms that oxidize carbohydrates (aerobic utilization) such as *Pseudomonas aeruginosa,* form those organisms that ferment carbohydrates (anaerobic utilization) such as members of the *enterobacteriaceae.*

Principle: The test organisms utilize carbohydrate in both the open and sealed tubes as shown by a change in colour of the medium form green to yellow. Oxidative organism, however able to use the carbohydrate only in the open tube. There is no carbohydrate utilization in the sealed tube (medium remains green). Although most genera of aerobics are either carbohydrate oxidizers or fermenter, the productions of acid may be slow and therefore cultures are usually incubated for 7-14 days.

Some bacteria are unreactive in the conventional O-F medium, either because they are unable to grow in the basal medium or because they cannot attack glucose.

Requirements : cultures of *E.coil* and *Pseudomonas aeruginosa,* O-F medium, sterile liquid paraffin (paraffin oil) nichrome straight wire.

Composition (O-F medium)

Tryptone or peptone	2.0 g
Sodium chloride	5.0 g
di-potassium hydrogen phosphate anhydrous (H2KPO4)	0.3 g
Agar	2.5 g
Gromothymol blue, 10g/1 (1% w/v)	3.0 ml
Distilled water	1.01

Prepared by dissolving 0.5 in 50 ml of distilled water.

1. Mix the dry ingredients in the water and heat to 100°C to dissolve (place the flask in a container of boiling water.) adjust the pH to7.1.
2. Allow to cool to 50-55°C and then add 3 ml of the bgromothymol blue indicator. Mix well.
3. Dispense in 50 ml amounts in screw-cap containers. Sterilise by autoclaving (with caps loosened)at 121°C for 15 minutes.
4. Cool to 50-55°C and then add aseptically 5 ml of a sterile 10 % w/v glucose (dextrose) solution. Mix well.
5. Dispense aseptically in 9 ml amounts in sterile screw-cap tubes or bottles.

Procedure:

1. Using a sterile straight wire, inoculate the test organism to the bottom of two tubes of sterile O-F medium. Use a heavy inoculum. Before inoculation heat the tubes in a beaker of boiling water bath to drive off any dissolved oxygen.

2. Cover the inoculated medium in one of the tubes with a 10 mm deep layer of sterile paraffin oil to seal it from air.

3. Incubate the tubes at 35-37°C for up to 14 days. Examine daily for carbohydrates utilizations as shown by acid production.

Interpretation:

Open tube	Sealed tube	Interpretation	Example
Yellow	Green	Oxidative organism	*Pseudomonas spp.*
Yellow	Yellow	Fermentative organism	*E.coli*
Green or blue	Green	No utilization of carbohydrates	*Bordetella spp.*

Note: This medium can also be used for detecting gas production and motility.

Sugar fermentation test

Principle: certain bacteria attack a characteristic range of carbohydrates producing acidity. Detercted by an indicator, and sometimes gas, detected by a submerged, inverted durham tube.

Constituents of fermentation test media

1. A suitable nutrient medium, such as peptone water, as a base to allow the growth of the organism under test, free from substances that might yield acid products. The nature of this medium depends on the nutritional requirements of the organism. Peptone water, serum peptone water and serum agar are commonly used.
2. The carbohydrates or related compound under test. A large variety is used and they are often referred to loosely as „sugars.
3. A suitable indicator that will change colour only as a result of the formation of acids during the fermentation. Some common examples are Andrade‟s indicator (used at a final concentration of 0.005%), bromocresol purple (used at a final concentration of 0.005%), phenol red (used at a final concentration of 0.01%), and bromothymol blue (used at a final concentration of 0.0025%). Bromothymol blue is used in preference to Andrade‟s indicator because it does not fade quickly.

4. A small inverted tube, known as Durham tube completely filled with liquid and containing no air bubbles is usually included in each culture tube or bottle to detect gas.

Requirements: peptone water culture of E. *coli and S. typhimurium,* sterile peptone water fermentation test media with different sugars, Durham tube, sterile pasture pipette.

Composition

Peptone water sugar

Peptone	10.0g
Sodium chloride	5.0g
Bromothymol blue, 2g/l (0.2% w/v)	12.5ml
Distilled water	1000.0ml

Prepared by dissolving 0.1g of bromothymol blue in 2.5 ml of N/10 sodium hydroxide. Add 47.5 ml of sterile distilled water and mix well. Store in the dark.

1. Dissolve the peptone and salt in the water. Adjust the pH to 7.2-7.3. Add the indicator solution.
2. Dispense in 100 ml amounts in screw-cap bottles and sterilze by autoclaving (with caps loosened) at 121°C for 15 min. when cool, tighten the bottle caps.

5% peptone water sugar

Sterile peptone water with indicator	100.0ml
Sterile 10 % w/v sugar solution	5.0 ml

Prepared by dissolving 2.5 g of the sugar in 100 ml of distilled water and sterilized by filtration or steaming for 30 min alternatively, buy the ampoules containing 5 ml of sterile 10 % sugar solution or discs impregnated with sugar from commercial suppliers.

1. Add aseptically the sterile sugar solution to the sterile peptone water-containing indicator, and mix well.
2. dispense aseptically in 4 ml amounts in sterile Bijou bottles containing an inverted Durham tube (sterilized with the bottle) the pH of medium should be near pH 7.6, which will give the medium a blue colour; bromothymol blue indicator becomes yellow at pH6.0.

Procedure

1. Lable sugar tubes properly in a rack (3 each).
2. With the help of a sterile Pasteur pipette, inoculate one tube of each sugar with two drops of *E.coli* culture.
3. Repeat with culture of *S. Typhimurium* keep third tube of each sugar as uninoculated control.
4. Incubate the tubes at 37°C for at least 48 h loner if required.

Interpretation

Yellow	acid produced
Blue	acid not produced (no colour change)

Gas is detected as the air bubble in the inverted Durham tube.

Methyl red test

Principle: This is employed to detect of sufficient acids during fermentation of glucose resulting in pH below 4.5 and its sustained maintenance during period of incubation. Consequently upon addition of methyl red indicator (pH range: 4.0-6.2) red colour appears in the reaction tube. The test is preformed to differentiate members of the family *Enterrobacteriaceae*. As organisms may further metabolize the acids to various neutral products, the duration of incubation period is very important.

Requirements: cultures of *E. coli* and *Klebsiella,* glucose phosphate peptone water tubes, methyl red indicator.

Glucose phosphate water

Peptone	0.5g
Glucose (destrose)	0.5g
di- potassium hydrogen phosphate (K_2HPO_4)	0.5 g
Distilled water	100.0ml

1. Dissolve the peptone and phosphate salt in the water by steaming. When cooled, filter and adust the pH to 7.5.
2. Add the glucose and mix well.
3. Dispense the medium in 1.5 ml amounts in small screw-cap tubs or bottles.
4. Sterilize by autoclaving (with caps loosened) at 115°C for 10 min. when cool, tighten the container tops.

Methyl red indicator

Methyl red	0.1g
Ethanol	300.0 ml
Distilled water	200.0 ml

Procedure

1. Inoculate one tube of phosphate peptone water with *E.coli* culute and the other with kleb. *Pneumonia.* Keep third tube as control.
2. Incubate all tubes at 37°C for 48 hours
3. Add 5 drops of methyl red indicator in each culture tube and tube also in one uninoculated control tube for comparing the colour.

Interpretation:

Red	Positive
Yellow	Negative
Orange	Doubtful

Note: the result after 48 h are suspicious the test should be repeated

Voges – Proskauer test

Principle : Certain bacteria ferment carbohydrates to produce acetyl methyl carbinol (CH_3 CHOH.CO.CH_3) or its reduction product 2,3 butylene glycol (CH_3 CHOH.CO.CH_3) from pyruvic acid in the medium, which can be detected by colorimetric methods. Following addition of alkali and then vigorous shaking of the tube, acetyl methyl carbinol or 2,3 butylene glycol is oxidized to diacetyl. Diacetyl reacts with the guanidine or arginine present in the medium (peptone) and produces a pink red colour.

The test is usually done in conjunction with the methyl red test since the productionof acetyl methyl carbinol or butylenes glycol usually result in insufficient acid accumulation during fermentation to give a methyl red positive reaction. An organism of the Enterobacteriaceae family group is usually either MR positive or V.P negative or vice-versa.

Method 1

Requirement: cultures of *E.coli* and *klep. Pneumonia,* glucose phosphate peptone water, 5% alcoholic solution of α- naphthol and 40 % aqueous solution of potassium hydroxide.

Procedure:

1. Inoculate one tube of glucose phosphate peptone water with *E.coli* culture and the other with *kelb. Pneumonie.*
2. Incubate the inoculated tube at 37°C for 48 to 72 h.
3. Add 0.6 ml of α-naphthol and 0.2 ml of postassium hydroxide solution to ml of each culture. Shake thoroughly, slope the tube and examine after 15 min add 1 h.

Modified O'Meara method

Requirements : cultures of *E.coli* and *Kleb Pneumonia* glucose phosphate peptone water, 40% aqueous solution of sodium hydroxide, creatinine powder.

1. Inoculate 2 ml of sterile glucose phosphate peptone water with the test organism.
2. Incubate at 35-37°C for 48 h.
3. Add very small amount (knife point) of creatinine and mix.
4. Add about 3 ml of the sodium hydroxide reagent and skake well.
5. Remove the plug and leave at room temperature. Look for the slow developments of a pink-red colour after 1 h and 4h.

Note: instead of creatinine powder and sodium, hydroxide, some workers recommend adding 2 drops (about 0.05 ml) of 1 % creatinine solution (v/v) in 0.1 NHCL and 1 ml of 40 % postassium hydroxide solution.

Interpretation:

Pink-red	positive test (acetioin produced)
No pink- red colour	negative test (no acetioin produced)

Citrate utilization test

This test is performed for the identification of *enterobacteria*.

Principle: The test organism is cultured in a medium containing sodium citrate, ammonium salt and bromothymol blue indicator. The organisms use citrate (the only source of carbon) and ammonia (the only source of nitrogen) .The citrate utilization is followed by alkaline reaction (change of the colour from light green to blue) and growth in the medium is indicated by appearance of turbidity.

Requirements: cultures of *Kleb. pneumonia* subsp *aerogenes* and *E. coli* citrate medium, inoculating wire and loop.

Koser"s method

Composition

Sodium chloride	5.0 g
Magnesium sulphate, $MGSO_4$	0.2 g

Ammonium dihydrogen phosphate $NH_4H_2PO_4$	1.0g
Postassium dihydrogen phosphate KH_2PO_4	1.0g
Sodium citrate $NA_3\ C_6H_5O_7\ 2H_2\ O$	5.0 g
Distilled water	1.0l

Adjust the pH to 6.8 and sterilize by autoclaving at 121°C for 15 min; the medium should be colourless.

Procedure: inoculate medium and incubate at 37°C Examine daily for upto 7 days A Positive test should be confirmed by sub cultureing again to same medium.

Interpretation:

Turbidity	Positive test
No turbidity	Negative test

Simmons' method

Composition

Kesor"s medium	1.01
Agar	20.0g
Bromothymol blue 0.2 % solution	40.0 ml

Dispense, autoclave at a single streak lightly over the surface of slant and incubate at 37°C examine daily for upto 7 days growth and colour change. Confirm positive result by subculturing to the same medium

Interpretation:

Blue colour and streak of growth	Positive test
Original green colour and no growth	Negative test

Christensen method

Composition:

Sodium citrate	3.0g
Glucose	0.2g
Yeast extract	0.5g
L- cysteine HCL	0.1g
KH2PO4	1.0g
Sodium chloride	5.0g
Agar	20.0g
Phenol red, 0.2% aqueous soln.	6.0 ml
Distilled water	100.0 ml

Dissolve, adjust pH to 6.8-6.9, autoclave at 115°C for 20 min and set as slope.

Procedure: inoculate medium by stabbing the butt and then drawing the wire over the surface of the slope. Incubate at 37°C examine daily for colour change.

Interpretation:

Magenta colour	Positive test
Yellow colour	Negative test

Note: An organism giving positive result in Koser"s or simmon"s method will be positive in Christensen medium, but reverse may or may not be positive.

Note: For citrate utilization test all glassware must be chemically clean and alkai free. For this boil all tubes and flasks in 20 % nitric acid for 5-10 min and than wash and rinse well with glass distilled water keeping tubes in the inverted position in baskets lined with blotting paper (to prevent the mouths of the tubes touching the metal) and sterilize in a hot air over at 160-170^{+}C for 1 h.

TESTS FOR METABOLISM OF PROTEINS AND AMINO ACIDS

The naturally occurring proteins are too large to enter the bacterial cell. They are first degraded to smaller components by the process of proteolysis by extracellular enzymes secreted by bacteria. Proteolysis occurs in two stages. In the first stage proteinases degrade proteins into smaller molecules, called polypeptides, and in the second stage polypeptides are degraded into a mixture of individual amino acids with the help of peptidases enzymes. The cell without any breakdown may use the amino acids or the process of deamination and decarboxylation may degrade them.

Proteolytic organisms digets proteins and consequently may liquefy gelatin or coagulated serum. Liquefaction of gelatin, being the commonest proteolytic property, is used routinely as an index of proteolytic activity but a positive result may take several days to develop. Cultures in meat media cause blackening of the meat, decomposing it and reducing its volume with formation of foul-smelling products. Some organisms decompose milk proteins whereas strongly proteolysis organisms may have all these properties, weakly proteolytic ones may liquefy only gelatin.

Principle : Gelatin is a protein , which is liquefied by gelatinase enzyme produced by some bacteria. Liquefied gelatin cannot form a gel. The criterion of gelatin liquefaction helps in the identification of certain species of bacteria.

Requirements : culture of *Protaus* spp. and *E. coli*, gelatin tubes, nichrome straight wires.

Composition

Galatin medium

Gelatin	120.0g
Distilled water	1000.0ml

1. Dissolve gelatin in 100 ml of distilled water by heating.
2. Dispense in 7 ml amounts in test tubes (15x125 mm)
3. Sterilize by autoclaving at 115°C for 20 min.

Nutrient Gelatin

Meat extract	3.0g
Peptone	5.0g
Gelatin	120.0g
Water	1000.0 ml

Add the gelatin to the water and allow standing for 15-30 min.

1. Heat to dissolve the gelatin; do not ingredients.
2. Add and dissolve the other ingredients.
3. Adjust to pH 7.0 and dispense in test tubes.
4. Sterilise by autoclaving at 115°C for 20 min.

Procedure

1. Inoculate the gelatin tube with proteus spp. And the other tube with E.coil by stabbing using a straight wire. The third tube will act as uninoculated control.
2. Incubate all these tubes at 37°C for upto 14 days; incubation at 20°C for longer period is preferred if the organisms can grow at this temperature.
3. After every 2-3 days place all gelatin tubes in a refrigerator for 2 h or till the medium of the control tube solidifies. Compare the medium of the inoculated tubes with that of control tube.

Interpreation: liquefied gelatin does not solidify on refrigeration.

Digestion of milk (casein hydrolysis)

Requirements: cultures of *Bacillus subtilis* and *Escherichia coli*. Casein milk agar.

Composition

Nutrient agar, sterile	87.5 ml
Skimmed milk, sterile	12.5 ml

(sterilized at 115°C for 10 min)

Melt the agar, cool to 50°C add the milk and pour plates

Procedure: Divide the skim milk agar plate in half and inoculate one half with *Bacilus subtilis* and the other half with *Escherichia coli.* Incubate at 37°C for 10-14 days.

Hydrogen sulphide production

Principle:- production of hydrogen sulphide by the microorganisms is tested in the medium containing sulphur compounds like cystine, methionine and glutathione. Bacteria may be grown in ordinary broth, peptone water or in any other medium containing sulphur compounds hydrogen sulphide produced by the breakdown of sulphur compounds can be detected by the incorporation of a heavy metal salt(e.g., lead, iron, etc.) into the medium Hydrogen sulphide reacts with the metal to form black metal sulphides. The production of H2S is usually detected by hanging a lead acetate filter paper strip in the culture tube. Hydrogen sulphide liberated from the medium will act on the lead acetate filter paper changing it swhite colour to black.

For H2S production excessive aeration in the culture should be avoided.

Requirements: cultures of *E.coli* and *Proteus vulgaris*, nutrient broth or peptone water tubes, sterile filter paper strips saturated with lead acetate. Preparation of strips

1. Cut 5-10 mm (wide) x 50-60 mm (long) strips of filter paper.
2. Soak the trips in lead acetate (tri-hydrate salt) hot saturated aqueous solution (10g) lead acetate in 100 ml hot water.
3. Dry at 50 to 60°C and store in airtight containers.

Procedure: inoculate one tube with *E.coli* and another tube with *Proteus vulgaris.* Insert a strip of lead acetate filter paper in between the side of the tube and cotton plug so that it hangs freely into the tube upto the height of about an inch from the top of the medium. Incubate at 37°C for 10-14 days

Interpretation: Hydrogen sulphide production will turn the paper brown or black by converting lead acetate to lead sulphide.

Indole Test

Principle: Bacteria utilize various amino acids as their food. Bacteria use the enzyme tryptophanase to convert the amino acid tryptophanase, the formation of indole from a tryptophan substrate can be another useful diagnostic tool for the identification of an organism. Indole production is a key test for the identification of *Escherichia coli.*

Testing for indole production is important in the identification of enterobactera. Four metabolic tests, viz, in dole production, methyl red test, voges- proskauer reaction and citrate utilization, collectively known as IMViC tests, are used to differentiate members of Enterobacteriaceae family.

Ehrlich's method

Requirements: culture of *E.coli* and *kleb. Pneumonia,* petone water tubes, either Ehrlich"s reagent.

Composition

Para- dimethyl amino benzaldehyde	1.0g
Ethanol, absolute	95.0g
Conc .HCI `	20.0 ml

Dissolve the aldehyde in the ethanol and add the acid

Or

Para-dimethyl amino benzaldehyde	2.0g
Conc.HCI	20.0ml
Distilled water	80.0ml

Procedure

1. Inoculate one tube of peptone water (5 ml) with *E.coli* and the other with *Kleb.pneeumoniae.*
2. Incubate both tubes at 37°C for 48 hrs.
3. Add 1 ml of ether of each tube. Shake well and allow standing till either collects on the surface of the medium.
4. Run down 1 ml of Ehrilch"s reagent by the side of the tube and watch for the development of deep red colour.

Interpretation: The presence of indole is indicated by the development of deep red colour at the interface of the reagent and ether

Kavac's method

Requirements: peptone water tubes, cultures of *E.coli* and *Kleb. Pneumonia* Kovac"s reagent.

Composition

p-dimethyl amino benzaldehyde	5.0g
amyl or isoamyl alcohol	75.0ml
conc.HCI	25.0ml

dissolve the aldehyde in the alcohol and slowly add the acid . Store in brown bottle at 4°C.

procedure

1. Inoculate one peptone water tube with *E.coli* and another with *kleb. Pneumoniae*
2. Incubate at 37°C for 48h.
3. Add 0.5 ml kovac‟s and shake gently.

Interpretation: A red colour in the alcohol layer indicates a positive reaction.

Urease test

Principle: some bacteria produce the enzyme urease, which breakdown the urea (by hydrolysis) present in the medium to give ammonia and carbon dioxide. With the release of ammonia, the medium becomes alkaline due to which the color of the indicator (phenol red) added in the medium change from yellow to pink or red. Urease test is useful for differentiating enterobacteria. *Proteus Strains* are urease positive while S*almonellae and Shigellae* do not produce this enzyme.

Requirements: cultures of *Proteus* and *Salmonella* and urea agar.

Composition

Peptone	1.0g
Sodium chloride	5.0g
Potassium monohydrogen (or dihydrogen)phosphate	2.0g
Agar	20.0g
Distilled water	1.01

Dissolve the ingredients by heating; about the pH to 6.8 and autoclave at 121°C for 15 min. and cool to 50°C.

Glucose	1.0g
Phenol red (0.2%)	6.0ml

Add to the molten base, steam for 1 h and cool to 50°C.

Usera 20% soln. (Sterilised by fitration)	100.0ml

Mix well and prepare slants.

Procedure

1. Inoculate one slant with *Proteus* and other with *Salmonella.*
2. Incubate at 37°C for 24 to 48 hr.

Ex 10 ANTIMICROBIAL SUSCEPTIBILITY TESTING

Objective: In vitro antimicrobial sensitivity of given a bacterial culture.

Materials: Test culture, Muller – Hinton agar, Tryptone soyabroth, 0.5 farland turbidity standard, sterile non-toxic cotton swabs antibiotic discs, forceps.

Muller-Hinton agar

Beef in fusion	300 .00g
Casein acid hydrolysate	17.50g
Starch	1.50g
Agar	1.07g
Distilled water	1.00l.

Emulsify the starch in a small amount of cold water, mix beef infusion and other ingredients. Make up the the volume to 1 litter. Dissolve by heating. Adjust the pH to 7.5. sterilse by autoclaving at 121°C for 15 min. Pour the plates up to depth of 4 mm check for sterility before using.

Typtone soya broth

Casein enzymic nydrolysate	17.5g
Papain digest of soybean meal	3.0g
Sodium chloride	5.0g
Dipotassium phosphate	2.5g
Glucose	2.5g
Distilled water	1.0l

Dissolve the ingredients in water by gentle heating. Adjust the pH to 7.3 sterilize by autoclaving at 121 °C for 15 min. Distribute in test tubes in 5 ml check for sterility before using.

McFarland 0.5 turbidity standard

Solution A (0.048 M $BaCl_2$)

BaCL2H2O	1.175g
Distilled water	100.000ml
Solution B	(0.18m H_2SO_4)
H_2SO_4(A.R grads, Sp.gr.1.84)	1.0ml
Distilled water	100.0ml
0.5 Standard	
Solution A	0.5ml
Solution B	99.5ml

Shake vigorously and dispense into 4-6 ml sealed tubes or screw-capped vials. Stored in the dark at room temperature and use upto 3 months. The turbidity should always be agitated before use.

Procedure

1. Select 4-5 well- isolated and identical bacterial colones grown overnight on a non-selective medium, such as Nutrient agar or Blood agar. Transfer these colonies into a tube of sterile tryptone soya broth (TSB) (5ml).
2. Incubate the broth at 35-37°C for 2-8 h or until alight to moderate turbidity develops. Note : Altermatively the colonies may be directly suspended in sterile normal saline or TSB to prepare suspension of equivalent turgidity without incubation .This is suggested particularly for testing staphylococci.
3. Compare the turgidity of the pre-incubated broth or the saline suspension of bacteria with 0.5 McFarland turbidity standards against a white background with contrasting black lines. Adjust the turgidity of the test suspension, if required with sterile broth or saline, until it equates to that of the turbidity standard, if turbidity is less incubate the suspension for more duration.
4. Dip a sterile non-toxic cotton swab on applicator stick into the standardized bacterial suspension and express excess fluid by pressing and rotating the swab against the inside wall of the tube.
5. Streak the swab in three directions over the entire surface of a sterile, dry plate of Mueller-Hinton agar, turning the plate at 60°C angle between each streaking, so as to obtain a uniform growth.
6. Make a final sweep with the swab around the rim of the Petri dish.
7. Keep the plate at room temperature for 5-15 min to allow the excess moisture from the inoculums to be absorbed by the agar.
8. Place the antibiotic discs at equidistance (min 24 mm apart from centre to centre) on to the agar surface using a sterile forceps or an antibiotic disc dispenser Each disc should be gently pressed with the point of a sterile forceps.

Note

a) Alter placing each disc sterilize the forceps in flame

b) Maximum 6 discs can be accommodated in a 9 cm dimeter Petri plate (24 mm apart).

c) In case of fastidious bacteria and for penicillin and cephalosporins. The discs should be placed 30 mm apart from centre-to- centre, i.e. 4 discs per 9 cm Petri plate.

9. Incubate the plate aerobically at 35-37°C in an inverted position for 16-18 (24 h for *Staphylococci*) or as recommended by the manufacturer.

   Note the plats should be shifed in to the incubator within 15 min. of applying the discs.

10. Read the diameter of zones of inhibition around each disc from the back of the plate to the nearest mm using a ruler the diameter of the zones should be read across the centre of the discs.

Results

S.No	Antibiotic	Symbol	Concentration	Zone of inhibition (in mm)

Exercise No. -11

Experiment: To study the morphological, cultural and biochemical characteristics of *staphylococcus aureus*.

Culture: Slant of known culture of *Staphylococcus* aureus

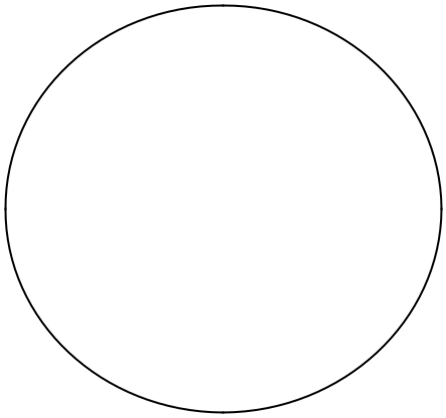
Materials:

1. Bunsen burner/spirit lamp, bacteriological loop, bacteriological slides, test tubes
2. Gram‟s staining set
3. Media: Nutrient broth, nutrient agar, blood agar, SM 110
4. Rabbit/bovine plasma
5. Media for biochemical tests and reagents

(A) Morphological characteristics

1. Take a grease-free slide and mark tow lines on the slide at least one inch apart with the help of glass marking pencil.
2. Make the smear of given culture and fix it by passing the slide over the flame. Prevent over heating of slide.
3. Stain the smear with gram‟s staining method and remove excess of moisture by blotting.
4. Air-dry the smear and examine under oil immersion objective.
5. Note the morphological and staining characters of given culture.

Result:



(Fig: Gram-positive cocci in grape-like clusters)

(B) Cultural characteristics

1. Inoculate the given culture in sterile nutrient broth with the help of sterile platinum loop to see pigment production, turbidity and pellicle formation, it any.
2. Streak the given culture over sterile nutrient and blood agar plates to see the colony characteristic and type of haemolysis produced by the organism.
3. Incubate the inoculated broth and culture plates at 37^{o} C for 18 to 24 hours and record the results.

Result/Observations:

Sl. No.	Media	Observations
1	Growth in nutrient both	
2	Growth on nutrient agar plate	
3	Growth on blood agar plate	
4	Type of haemolysis on blood agar plate	

(C) Biochemical characteristics

1. Inoculate the given culture in the following media with the help of sterile platinum loop for performing various biochemical tests.
2. Incubate the tubes at 37^{o} C for 18 to 24 hours and record the results.

Results/Observations:

Sl. No.	Name of Media	Biochemical tests	Results
1	Glucose phosphate peptone water (GPPW)	MR test	
2	Glucose phosphate peptone water (GPPW)	VP test	
3	Peptone water	Indol test	
4	Nitrate agar slant	Nitrate reduction test	
5	Rabbit plasma (1:5)	Coagulase test (a) Tube test (b) Slide test	
6	Gelatin slant	Gelatin liquefaction	

(D) Catalase test

Take a drop of 3% hydrogen peroxide solution (freshly prepared) and pour it on a slide containing the culture or directly on the culture plate. In positive cases, the bubbling is seen due to breakdown of H_2O_2 by catalase enzyme produced by bacteria

$$2H_2O_2 + O_2 \xrightarrow{\text{Catalase}} 2H_2O_2 + O_2 \Uparrow \text{bubbles}$$

(E) Sugar fermentation reactions:

1. Inoculate the given culture in the following 1% sterile sugar media tubes.
2. Incubate the inoculated sugar tubes at 37^{o} C for 24 to 48 hours and note down the results in terms of fermentation of sugar by the given culture (acid or alkali).

Result/Observation:

Sl. No.	Name of sugar	Result (acid/alkali)
1	Glucose (aerobically)	
2	Glucose (anaerobically)	
3	Manitol (aerobically)	
4	Manitol (anaerobically)	
5	Lactose (aerobically)	
6	Sucrose (aerobically)	

(F) Conclusion

Genus: Streptococcus

The streptococci are widely distributed in nature and are commensals in man and animals. There are more than 37 species of streptococci, which are listed in bergey‟s manual of systematic bacteriology volume 2. The genus streptococcus includes all those bacteria, which are usually spherical (0.5 to 1 µ in size). Occur in chains of variable length and multiply in one plane. They are non-sporulated, non-flagellated and non-capsulated organisms. All the species of genus are gram-positive cocci arranged in short or long chains attached with plasmodesma. Sometimes, they are also found as single coccus or in pairs. They are usually aerobic but most of them are facultative anaerobe. Some strict anaerobic streptococci are also known. They are catalase and oxidase negative and ferment sugars.

In general, they produce pinpoint, dewdrop-like colonies on agar media preferably in media enriched with blood. They produce long chains in liquid media. Some strains are also haemolytic. Potentially pathogenic and non-pathogenic species of streptococci are commonly found over the skin and mucous membrane of genital, upper respiratory and digestive tracts of man and animals. These organisms are also isolated from mil as well as from faeces of man and animals.

Exercise No. – 12

Experiment: To study the morphological, cultural and biochemical characteristics of *staphylococcus pyogenes*.

Culture: Slant of known culture of *Staphylococcus pyogenes*

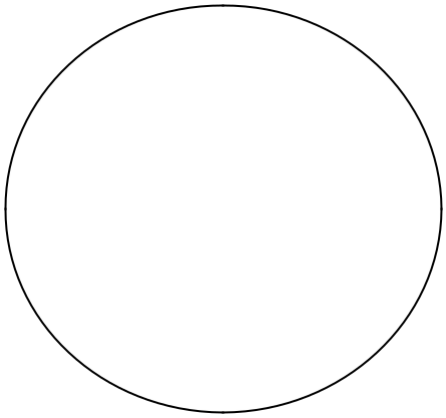
Materials:

1. Bunsen burner/spirit lamp, bacteriological loop, bacteriological slides, test tubes
2. Gram‟s staining set
3. Media: Nutrient broth, nutrient agar, blood agar
4. Media for biochemical tests and reagents

(A) Morphological characteristics

1. Take a grease-free slide and mark tow lines on the slide at least one inch apart with the help of glass marking pencil.
2. Make the smear of given culture and fix it by passing the slide over the flame. Prevent over heating of slide.
3. Stain the smear with gram‟s staining method and remove excess of moisture by blotting.
4. Air-dry the smear and examine under oil immersion objective.
5. Note the morphological and staining characters of given culture.

Result:



(Fig: Gram-positive cocci in grape-like clusters)

(B) Cultural characteristics

1. Inoculate the given culture in sterile nutrient broth with the help of sterile platinum loop to see pigment production, turbidity and pellicle formation, it any.
2. Streak the given culture over sterile nutrient and blood agar plates to see the colony characteristic and type of haemolysis.
3. Incubate the inoculated broth and culture plates at 37^{o} C for 18 to 24 hours and record the results.

Result/Observations:

Sl. No.	Media	Observations
1	Growth in nutrient both	
2	Growth on nutrient agar plate	
3	Growth on blood agar plate	
4	Type of haemolysis on blood agar plate	

(C) Biochemical characteristics

1. Inoculate the given culture in the following media with the help of sterile platinum loop for performing various biochemical tests.
2. Incubate the tubes at 37^{o} C for 18 to 24 hours and record the results.

Results/Observations:

Sl. No.	Name of Media	Biochemical tests	Results
1	Glucose phosphate peptone water (GPPW)	MR test	
2	Glucose phosphate peptone water (GPPW)	VP test	
3	Peptone water	Indol test	
4	Nitrate agar slant	Nitrate reduction test	

(D) Catalase test

Take a drop of 3% hydrogen peroxide solution (freshly prepared) and pour it on a slide containing the culture or directly on the culture plate. In positive cases, the bubbling is seen due to breakdown of H_2 O_2 by catalase enzyme produced by bacteria

(E) Sugar fermentation reactions:

1. Inoculate the given culture in the following 1% sterile sugar media tubes.
2. Incubate the inoculated sugar tubes at 37^{o} C for 24 to 48 hours and note down the results in terms of fermentation of sugar by the given culture (acid or alkali).

Result/Observation:

Sl. No.	Name of sugar	Result (acid/alkali)
1	Glucose (aerobically)	
2	Sorbitol (aerobically)	
3	Manitol (aerobically)	
4	Trehalose (aerobically)	
5	Lactose (aerobically)	
6	Salicin (aerobically)	

(F) Conclusion

Genus: Escherichia

Intestinal infections in man and animals caused by gram-negative and non-sportulated bacilli, particularly of genus Escherichia are collectively known as entrobacteria. They are classified in the family enterobacteriaceae. This family has more than 28 genera and 82 clearly defined species under it. Six genera of the family, viz., Escherichia, salmonella, citrobactor, klebsiella, proteus and shigella are of veterinary importance. The organisms placed under the genus Escherichia have worldwide distribution and are normal inhabitant of intestinal tract of man and animals. Some species are free living and found in soil and water. Faecal contamination of water is indicated by the presence of E. coli, Klebsiella, enterobacter and Citrobacter species, which have also been isolated from vegetables and food products. One of the important species of genus Escherichia is Escherichia coli, which is gram-negaative, non-sporulated and oxidase negative bacilli measuring 0.5 µm 1.0 µm to 3.0 µm is size. The shape may vary from coccoid or bipolar to long filamentous forms. Escherich first isolated the E. coli that produces acid and gas from glucose and lactose in the year 1885 from the faeccs of infants. It is usually motile but some species do not have flagella and are non-motile.

Exercise No. – 13

Experiment: To study the morphological, cultural and biochemical characteristics of *Escherichia coli*.

Culture: Slant of known culture of *Escherichia coli*

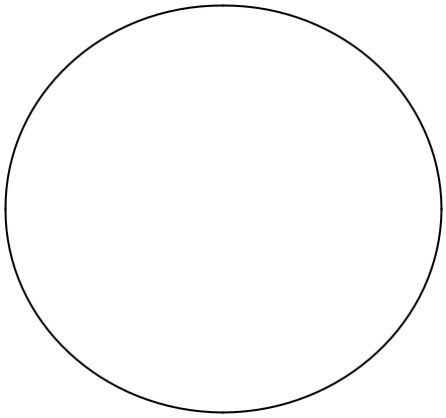
Materials:

1. Bacteriological slides, Bunsen burner/spirit lamp, bacteriological loop, test tubes
2. Gram‟s staining set
3. Media: Nutrient broth, nutrient agar, blood agar, MacConkey lactose age, (MLA), Brilliant green agar (BGA) plates Triple sugar iron slant (TSI)
4. Media for biochemical tests and reagents

(A) Morphological characteristics

1. Take a grease-free slide and mark tow lines on the slide at least one inch apart with the help of glass marking pencil.
2. Make the smear of given culture and fix it by passing the slide gently over the flame.
3. Stain the smear with gram‟s staining method and remove excess of moisture by blotting.
4. Air-dry the smear and examine under oil immersion objective.
5. Note the morphology and staining character of given culture.

Result:



(Fig: Gram-negative pleomorphic rods)

(B) Cultural characteristics

1. Inoculate the given culture in sterile nutrient broth with the help of sterile platinum loop to see pigment production, turbidity and pellicle formation, it any.
2. Streak the sterile nutrient agar, MacConkey lactose agar (MLA), EMB and Brilliant green agar (BGA) plates with the given culture to see the colony characteristic and other differential features of the organism.
3. Stab the TSI slant with the culture and also spread the culture over the slant to see the acid/alkaline reaction and H_2 S production by the organism.
4. Incubate the plates and tubes at 37^{o} C for 18 to 24 hours and record the results.

Result/Observations:

Sl. No.	Media	Observations
1	Growth in nutrient both	
2	Growth on nutrient agar plate	
3	Growth on MLA plate	
4	Growth on BGA plate	
5	Growth on TSI slant/butt	
6	Growth on EMB agar Metallic sheen present/absent	

(C) Motility: A drop of broth culture is put on a cover slip and examined for motility by hanging drop method in a cavity slide. Alternatively, the culture may be stabbed in the media containing semisolid agar and incubated at 37^{o} C for 24 hrs. Observe for spreading growth on either side of the stab.

Cavity slide method Stab culture method

(D) Biochemical characteristics

1. Inoculate the given culture in the following media for performing following biochemical tests.
2. Incubate the tubes at 37^{o} C for 18 to 24 hours.
3. Note IMViC pattern and other differential features of the given bacteria

Results/Observations:

Sl. No.	Name of Media	Biochemical tests	Results
1	Peptone water	Indol test	
2	Glucose phosphate peptone water (GPPW)	MR test	
3	Glucose phosphate peptone water (GPPW)	VP test	
4	Koser‟s citrate agar slant	Citrate test	
5	Triple sugar iron (TSI) agar slant	For H_2 S production	(a) Type of growth on slant (b) Type of growth on butt (c) H_2S production

(E) Sugar fermentation reactions:

1. Inoculate the given culture in the following 1% sterile sugar media tubes containing Durham‟s tube (for gas production).
2. Incubate the inoculated sugar tubes at 37^{o} C for 24 to 48 hours and note the fermentation reaction (acid or alkali) and production of gas by the given culture.

Result/Observation:

Sl. No.	Name of sugar	Result (acid/alkali)
1	Glucose (aerobically)	
2	Lactose (aerobically)	
3	Sucrose (aerobically)	

(F) Conclusion

Genus: Salmonella

Salmonella is most thoroughly studied genera of the family Enterobaeteriacease. It is worldwide in distribution and still causes substantial loss in health and production economy. There are more than 2000 serovars of *salmonella*. The salmonellae are gram-negative rods, 0.6 µm 3.0 to 4.9 µm in size and are closely related morphologically and physiologically to the other genera of the family. They are aerobic, usually motile, non-spore forming and noncapsulated organisms and produce acid and gas from glucose, maltose, manitol and sorbitol. These organisms do not ferment lactose, sucrose and salicin and are indol negative. They do not coagulate milk and fail to liquefy gelatin. Unlike other enterobacteria, the salmonellae are frequently facultative intracellular parasite. The invasive strains are taken up by macrophages and spread via the lymphatic pathway. Blood, or both. Infection of salmonellae is mainly by the oral route. The salmonellosis in humans is described as enteric fever, septicaemia and gastroenteritis. In animals, per acute septicaemia, acute sub acute and chronic enteritis are commonly seen. *Salmonella* possess (1) somatic antigen (O), (2) Flagellar antigen (ii) and (3) Capsular or envelope antigen (K). The Vi and M antigens are also seen in some serotypes. According to current taxonomy, the genus *salmonella* has only two species (I) *S. enterica* (II) *S. bongori* (WHO, 1997). About 99.5% of *salmonella* strains belong to *S. enterica* subspecies *enterica.* The serovars are written by first letter capital and are no longer italicized. For example *S. enterica* sub sp. Enterica serover Typhimurium is written as *S.* Typhimurium. Quite a strains of *salmonella* produce cytotonic and cytotoxic enterotoxins and cytotoxins.

Exercise No. – 14

Experiment: To study the morphological, cultural and biochemical characteristics of *Escherichia Bareilly*

Culture: Slant of known culture of *Escherichia Bareilly*

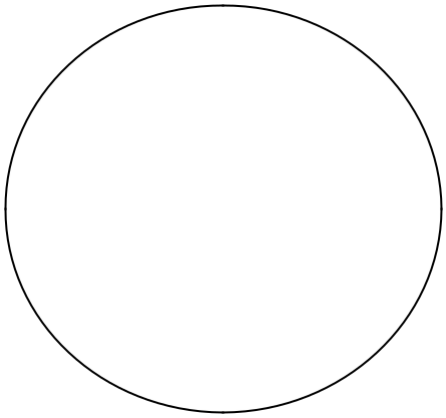
Materials:

1. Bacteriological slides, Bunsen burner/spirit lamp, bacteriological loop, test tubes
2. Gram‟s staining set
3. Media: Nutrient broth, nutrient agar, blood agar, MacConkey lactose agar, (MLA), Brilliant green agar (BGA) plates Triple sugar iron slant (TSI) slant.
4. Media for biochemical tests and reagents

(A) Morphological characteristics

1. Take a grease-free slide and mark tow lines on the slide at least one inch apart with the help of glass marking pencil.
2. Make the smear of given culture and fix it by passing the slide gently over the flame.
3. Stain the smear with gram‟s staining method and remove excess of moisture by blotting.
4. Air-dry the smear and examine under oil immersion objective.
5. Note the morphology and staining character of given culture.

Result:



(Fig: Gram-negative pleomorphic rods)

(B) Cultural characteristics

1. Inoculate the given culture in sterile nutrient broth with the help of sterile platinum loop to see pigment production, turbidity and pellicle formation, it any.
2. Streak the sterile nutrient agar, MacConkey lactose agar (MLA), EMB and Brilliant green agar (BGA) plates with the given culture to see the colony characteristic and other differential features of the organism.
3. Stab the TSI slant with the culture and also spread the culture over the slant to see the acid/alkaline reaction and H_2 S production by the organism.
4. Incubate the plates and tubes at 37^{o} C for 18 to 24 hours and record the results.

Result/Observations:

Sl. No.	Media	Observations
1	Growth in nutrient both	
2	Growth on nutrient agar plate	
3	Growth on MLA plate	
4	Growth on BGA plate	
5	Growth on TSI slant/butt	

(C) Motility: A drop of broth culture is put on a cover slip and examined for motility by hanging drop method in a cavity slide. Alternatively, the culture may be stabbed in the media containing semisolid agar and incubated at 37^{o} C for 24 hrs. Observe for spreading growth on either side of the stab.

Cavity slide method Stab culture method

(D) Biochemical characteristics

1. Inoculate the given culture in the following media for performing following biochemical tests.
2. Incubate the tubes at 37^{o} C for 18 to 24 hours.
3. Note IMViC pattern and other differential features of the given bacteria

Results/Observations:

Sl. No.	Name of Media	Biochemical tests	Results
1	Peptone water	Indol test	
2	Glucose phosphate peptone water (GPPW)	MR test	
3	Glucose phosphate peptone water (GPPW)	VP test	
4	Koser‟s citrate agar slant	Citrate test	
5	Triple sugar iron (TSI) agar slant	For H_2 S production	(a) Type of growth on slant (b) Type of growth on butt (c) H_2S production

(E) Sugar fermentation reactions:

1. Inoculate the given culture in the following 1% sterile sugar media tubes containing Durham‟s tube (for gas production).
2. Incubate the inoculated sugar tubes at 37^{o} C for 24 to 48 hours and note the fermentation reaction (acid or alkali) and production of gas by the given culture.

Result/Observation:

Sl. No.	Name of sugar	Result (acid/alkali)
1	Glucose (aerobically)	
2	Lactose (aerobically)	
3	Sucrose (aerobically)	

(F) Conclusion

Exercise No. – 15

Experiment: To study the morphological, cultural and biochemical characteristics of *Pseudomonas aeruginosa*

Culture: Slant of known culture of *Pseudomonas aeruginosa*

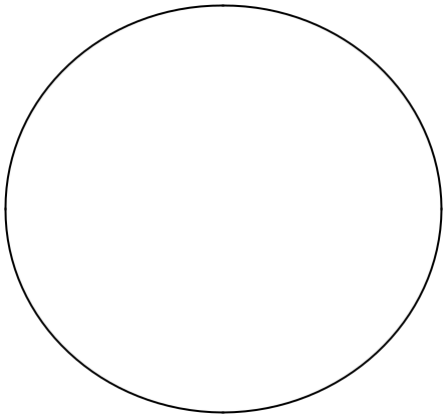
Materials:

1. Bunsen burner/spirit lamp, bacteriological loop, bacteriological slides, test tubes
2. Gram‟s staining set, capsule staining set, India ink
3. Media: Nutrient broth, nutrient agar, blood agar, MacConkey lactose agar, (MLA), Brilliant green agar (BGA) plates Triple sugar iron slant (TSI) slant.
4. Media and reagents for biochemical tests

(A) Morphological characteristics

1. Take a grease-free slide and mark tow lines on the slide at least one inch apart with the help of glass marking pencil.
2. Make the smear of given culture and fix it by passing the slide gently over the flame.
3. Stain the smear with gram‟s staining method and remove excess of moisture by blotting.
4. Air-dry the smear and examine under oil immersion objective.
5. Note the morphology and staining character of given culture.

Result:



(Fig: Gram-negative pleomorphic rods)

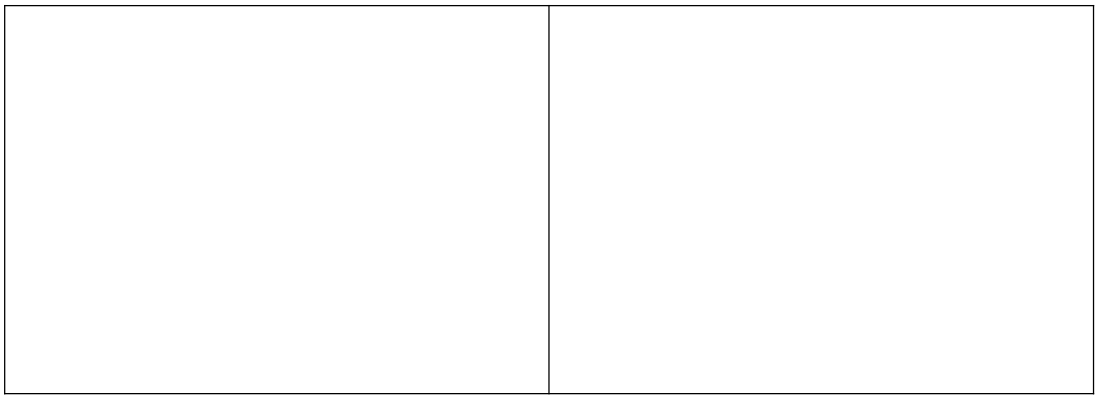
(B) Cultural characteristics

1. Inoculate the given culture in sterile nutrient broth with the help of sterile platinum loop to see pigment production, turbidity and pellicle formation, it any.
2. Streak the sterile nutrient agar, Cetrimide agar, SS agar, MacConkey lactose agar (MLA) and Brilliant green agar (BGA) plates with the given culture to see the colony characteristic and other differential features of the organism.
3. Stab the TSI slant with the culture and also spread the culture over the slant to see the acid/alkaline reaction and H_2 S production by the organism.
4. Incubate the plates and tubes at 37^{o} C for 18 to 24 hours and record the results.

Result/Observations:

Sl. No.	Media	Observations
1	Growth in nutrient both	
2	Presence/absence of pigments in NB	
3	Growth on nutrient agar plate	
4	Growth on MLA plate	
5	Pellicle formation in nutrient broth	
6	Growth on SS agar	
7	Growth on cetrimide agar	

(C) Motility: A drop of broth culture is put on a cover slip and examined for motility by hanging drop method in a cavity slide. Alternatively, the culture may be stabbed in the media containing semisolid agar and incubated at 37^{o} C for 24 hrs. Observe for spreading growth on either side of the stab.



Cavity slide method Stab culture method

(D) Biochemical characteristics

1. Inoculate the given culture in the following media for performing following biochemical tests.
2. Incubate the tubes at 37^{o} C for 18 to 24 hours.
3. Note IMViC pattern and other differential features of the given bacteria

Results/Observations:

Sl. No.	Name of Media	Biochemical tests	Results
1	Peptone water	Indol test	
2	Glucose phosphate peptone water (GPPW)	MR test	
3	Glucose phosphate peptone water (GPPW)	VP test	
4	Koser‟s citrate agar slant	Citrate test	
5	Triple sugar iron (TSI) agar slant	For H_2 S production	(a) Type of growth on slant (b) Type of growth on butt (c) H_2S production
6	Oxidase reagent (Tetra methyl	Oxidase test	

	p-phenylene diamine)		

(E) Sugar fermentation reactions:

1. Inoculate the given culture in the following 1% sterile sugar media tubes containing Durham"s tube (for gas production).
2. Incubate the inoculated sugar tubes at 37^{o} C for 24 to 48 hours and note the fermentation reaction (acid or alkali) and production of gas by the given culture.

Result/Observation:

Sl. No.	Name of sugar	Result (acid/alkali)
1	Glucose (aerobically)	
2	Gas from glucose	
3	Lactose (aerobically)	
4	Sucrose (aerobically)	
5	Maltose (aerobically)	

(F) Conclusion

Genus: Pasteurella

Microorganism of genus *Pasteurella* are gram-negative short rods usually cocciod or elongated cells measuring 0.25 to 0.4 µm X 0.6 to 2.6 µm in size. They are generally capsulated; however, non-capsulated forms do exist. Most of the species are non-motile, non-sporulated, aerobic to facultative anaerobic and exhibit characteristic bipolar staining. They are oxidase positive. The ability of *pasteurella* organisms to ferment carbohydrates is limited, although acid is produced in numbers of sugar media. Gas is not produced. Various species of genus *pasteurella* produce disease in man and animal. According to bergey‟s manual of systematic bacteriology, there are seven species of *pasteurella*, which are of importance to man and animals.

1. *Pasteurella multocida*: It occurs as commensals in the upper respiratory and digestive tracts of several species of animals.
2. *P. haemolytica*: It occurs as commensals in the upper respiratory and digestive tracts of several species of animals.
3. *P. pneumotropica*: It occurs as commensals in the upper respiratory and digestive tracts of several species of animals.
4. *P. ureae*: It is found in upper respiratory tract of human beings.
5. *P. gallmarum*: It is as commensals in upper respiratory tracts of chickens. Occasional low-grade respiratory infection may be seen in chickens.
6. *P. aerogenes*: It is also found as ocmmensals in the intestine of swine, but is rarely pathogenic.

Pasteurella multocida formerly called as *pasteurella* septica are the group of related bacteria that are isolated from haemorrhagic septicemia in a variety of animals and birds. Earlier they were known according to their species of origin: *P. boviseptica, P. lepiseptica, P. aviseptical* etc. though they show some degree of host specificity, they are so similar in other respects that they (*Pasteurella* spp.) are now considered as strains of single species, designated as *Pasteurella multocida* or *P multocida.*

Exercise No. 16

Experiment: To study the morphological, cultural and biochemical characteristics of *Pseudomonas multocida*

Culture: Slant of known culture of *Pseudomonas multocida*

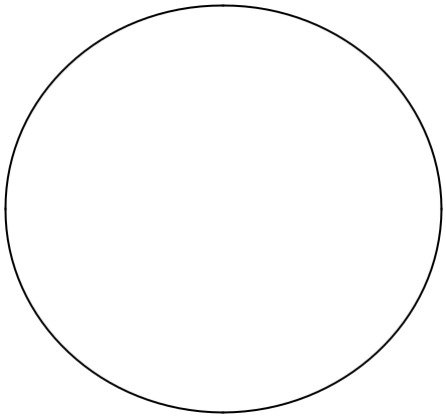
Materials:

1. Bunsen burner/spirit lamp, bacteriological loop, bacteriological slides, test tubes
2. Gram‟s staining set, capsule staining set, India ink
3. Media: Nutrient broth, nutrient agar, MacConkey lactose agar, (MLA) and blood agar plates.
4. Media and reagents for biochemical tests

(A) Morphological characteristics

1. Take a grease-free slide and mark tow lines on the slide at least one inch apart with the help of glass marking pencil.
2. Make the smear of given culture and fix it by passing the slide gently over the flame.
3. Stain the smear with gram‟s staining method and remove excess of moisture by blotting.
4. Air-dry the smear and examine under oil immersion objective.
5. Note the morphology and staining character of given culture.

Result:



(Fig: Gram-negative pleomorphic rods)

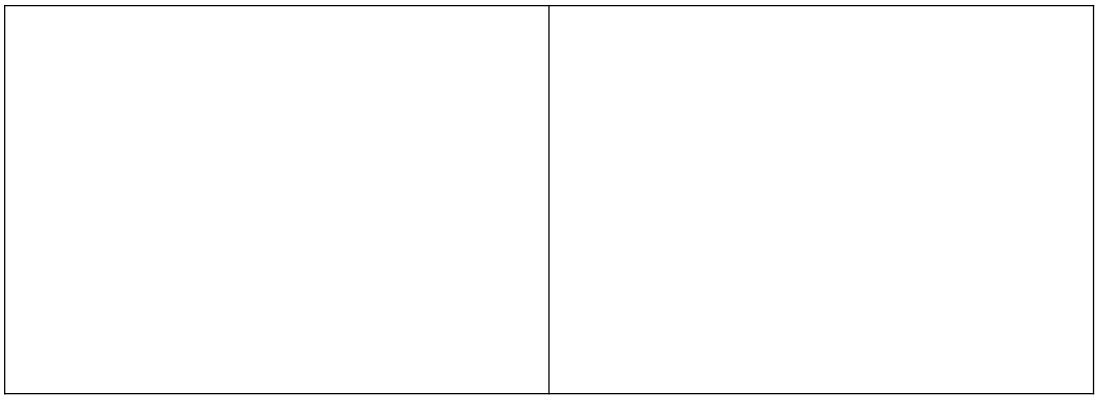
(B) Cultural characteristics

1. Inoculate the given culture in sterile nutrient broth with the help of sterile platinum loop to see pigment production, turbidity and pellicle formation, it any.
2. Streak the sterile nutrient agar, MacConkey lactose agar (MLA) plates with the given culture to see the colony characteristic and other differential features of the organism.
3. Stab the TSI slant with the culture and also spread the culture over the slant to see the acid/alkaline reaction and H_2 S production by the organism.
4. Incubate the plates and tubes at 37^{o} C for 18 - 24 hours and record the results.

Result/Observations:

Sl. No.	Media	Observations
1	Growth in nutrient both	
2	Pigments present/absence	
3	Growth on nutrient agar plate	
4	Growth on MLA plate	
5	Blood agar blafe	
6	Type of haemolysis on blood agar plate	
7	Motility at 22^{o} C	

(C) Motility: A drop of broth culture is put on a cover slip and examined for motility by hanging drop method in a cavity slide. Alternatively, the culture may be stabbed in the media containing semisolid agar and incubated at 37^{o} C for 24 hrs. Observe for spreading growth on either side of the stab.



Cavity slide method Stab culture method

(D) Biochemical characteristics

1. Inoculate the given culture in the following media for performing various biochemical tests.
2. Incubate the tubes at 37^{o} C for 18 to 24 hours.
3. Note IMViC pattern and other differential features of the given bacteria

Results/Observations:

Sl. No.	Name of Media	Biochemical tests	Results
1	Peptone water	Indol test	
2	Glucose phosphate peptone water (GPPW)	MR test	
3	Glucose phosphate peptone water (GPPW)	VP test	
4	Koser‟s citrate agar slant	Citrate test	
5	Growth in urea agar medium	Urea test	
6	Oxidase reagent (Tetra methyl p-phenylene diamine)	Oxidase test	

(E) Sugar fermentation reactions:

1. Inoculate the given culture in the following 1% sterile sugar media tubes containing Durham‟s tube (for gas production).
2. Incubate the inoculated sugar tubes at 37^{o} C for 24 to 48 hours and note the fermentation reaction (acid or alkali) and production of gas by the given culture.

Result/Observation:

Sl. No.	Name of sugar	Result (acid/alkali)
1	Glucose (aerobically)	
2	Gas from glucose	
3	Lactose (aerobically)	
4	Sucrose (aerobically)	
5	Maltose (aerobically)	

(F) Conclusion

Genus: Bacillus

Species of the genus *bacillus* are gram-positive and aerobic or facultative anaerobic large rods. They are spore forming cylindrical bacilli, which are usually flagellated and grow in long chaing on solid media like nutrient agar. They produce irregular. Medusa head colonies on agar plate. The members of genus *bacillus* are ubiquitous and found normally in soil, air, dust and water. These organisms are also found on decaying vegetation and are most active in bringing about the decomposition of organic substances, particularly those grouped together under the term ammonification. They are one of the most important organisms of soil fertility. They are also known as most common laboratory contaminants. The organisms spread by water, winds and transportation of feeds/feed ingredients. *Bacillus* organisms are usually flagellated and slightly curved rods with rounded ends and measure 1µm in diameter by 3 to 4 µm in length. Most of them are catalase positive, fermentative or respiratory or both. Some do not attack sugars. If clinical samples such as bovine milk are not collected carefully, it often gets contaminated with *bacillus* species. Because of the resistance of their spores to high temperatures and desiccation, these organisms are among those that frequently contaminate the laboratory media. *Bacillus anthracis* is the only important pathogen of animals and humans in the genus. *B. cereus* is also incriminated in number of animal diseases, but infections by other species is rare. *Bacillus subtilis* may cause iridocyclitis and panophthalmitis and may sometimes produce fatal septicaemia. It has been isolated in pure culture from liver and mandibular abscesses in cattle and sheep. *Bacillus megaterium*, in large doses, is capable of producing death in guinea pigs. Pseudoanthrax bacilli are able to kill mice and guinea pigs, but when used in large doses, it may be confused with *bacillus anthracis*. The differences between the two are given below:

Sl. No.	***Bacillus anthracis***	**Pseudoanthrax bacilli (Anthracoid)**
1	Non-motile	Generally motile
2	Capsulated	Non capsulated
3	Grows in long chains	Grows in short chains

4	No turbidity in nutrient broth	Produce turbidity in nutrient broth
5	Produces inverted fir tree like growth in gelatin media	Produces no fir tree growth in gelatin media (If produced, it is typical one)
6	Pathogenic for laboratory animals	Non pathogenic for laboratory animals
7	Liquefies gelatin slowly	Liquefies gelatin rapidly
8	Medusa head colony present	Medusa head colony absent
9	No growth on penicillin agar (10 units/mi)	Usually grown
10	Salicin fermectation agar negative	Usually positive
11	No growth at 45^{o} C	Usually grow
12	Growth inhibited by chloral hydrate	Not inhibited
13	Susceptible to gamma phage	Not susceptible

Exercise No. – 17

Experiment: To study the morphological, cultural and biochemical characteristics of *Clostridium perfringens*

Culture: Slant of known culture of *Clostridium perfringens*

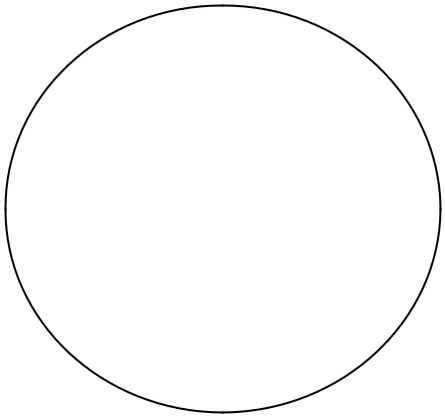
Materials:

1. Bacteriological slides, Bunsen burner/spirit lamp, bacteriological loop, test tubes cavity slide
2. Anaerobic jar (Novy‟s or MacIntosh and Fildes‟ Jar), hydrogen gas cylinder
3. Gram‟s staining set, methylene blue
4. Media: Nutrient broth, nutrient agar, blood agar, Robertson cooked meat media (broth & agar)
5. Media and reagents for biochemical tests

(A) Morphological characteristics

1. Take a grease-free slide and mark tow lines on the slide at least one inch apart with the help of glass marking pencil.
2. Make the smear of given culture and fix it by passing the slide gently over the flame.
3. Stain the smear with gram‟s staining and remove the excess of moisture by blotting.
4. Air-dry the smear and examine under oil immersion objective. Also see the type of spores, which may not be stained.
5. Note the morphology and staining character of given culture.

Result:



(Fig: Gram-positive rods)

(B) Cultural characteristics

1. Inoculate the given culture in sterile nutrient broth with the help of sterile platinum loop to see pigment production, turbidity and pellicle formation, it any.
2. Streak the sterile nutrient agar, blood agar and Robertson cooked meat media plates and infusion broth with the given culture to see the colony characters and other differential features of the organism including haemolysis.
3. Stab the TSI slant with the culture and also spread the culture over the slant to see the acid/alkaline reaction and H_2 S production by the organism.
4. Incubate the broth clant and plates at 37^{O} C for 18 - 24 hours in Novy‟s or MacCantosh jar anaerobically.
5. Record the growth characteristic and other differential features.

Result/Observations:

Sl. No.	Media	Observations
1	Growth in nutrient both (Anaerobically)	
2	Growth on nutrient agar plate (Anaerobically)	
3	Growth in Robertson cooked meat infusion broth (Anaerobically)	
4	Growth in Robertson cooked meat agar plate (Anaerobically)	
5	Demonstration of motility by cavity slide method	

6	Growth on blood agar plate (Anaerobically)	

(C) Motility: A drop of broth culture is put on a cover slip and examined for motility by hanging drop method in a cavity slide. Alternatively, the culture may be stabbed in the media containing semisolid agar and incubated at 37^{o} C for 24 hrs. Observe for spreading growth on either side of the stab.

Cavity slide method Stab culture method

(D) Biochemical characteristics

1. Inoculate the given culture in the following media for performing various biochemical tests.
2. Incubate the tubes at 37^{o} C for 18 to 24 hours under anaerobic conditions.
3. Note IMViC pattern and other differential features of the given bacteria

Results/Observations:

Sl. No.	Name of Media	Biochemical tests	Results
1	Peptone water	Indol test	
2	Glucose phosphate peptone water (GPPW)	MR test	
3	Glucose phosphate peptone water (GPPW)	VP test	
4	Koser‟s citrate agar slant	Citrate test	

5	Triple sugar iron (TSI) agar slant	For H_2 S production	(a) Type of growth on slant (b) Type of growth on butt (c) H_2S production
6	Growth in geratin medium	Gelatin liquefaction	
7	Nitrate medium	Nitrate test	

(E) Sugar fermentation reactions:

1. Inoculate the given culture in the following 1% sterile sugar media tubes containing Durham"s tube (for gas production).
2. Incubate the inoculated sugar tubes at 37^{o} C for 24 to 48 hours and note the fermentation reaction (acid or alkali) and production of gas by the given culture.

Result/Observation:

Sl. No.	Name of sugar	Result (acid/alkali)
1	Glucose (anerobically)	
2	Lactose (anerobically)	
3	Sucrose (anerobically)	
4	Gas from glucose	
5	Litmus milk	(a) Acid (b) Digested

(F) Conclusion

Exercise No. – 18

Experiment: To study the morphological, cultural and biochemical characteristics of *Clostridium chauvoei*

Culture: Slant of known culture of *Clostridium chauvoei*

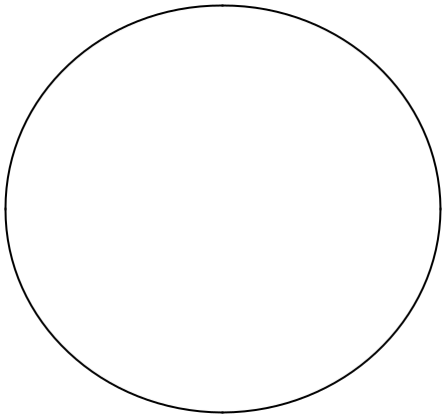
Materials:

1. Bacteriological slides, Bunsen burner/spirit lamp, bacteriological loop, test tubes cavity slide
2. Anaerobic jar (Novy‟s or MacIntosh and Fildes‟ Jar), hydrogen gas cylinder
3. Gram‟s staining set, methylene blue
4. Media: Nutrient broth, nutrient agar, blood agar, Robertson cooked meat media (broth & agar)
5. Media for biochemical tests and reagents

(A) Morphological characteristics

1. Take a grease-free slide and mark tow lines on the slide at least one inch apart with the help of glass marking pencil.
2. Make the smear of given culture and fix it by passing the slide gently over the flame.
3. Stain the smear with gram‟s staining and remove the excess of moisture by blotting.
4. Air-dry the smear and examine under oil immersion objective. Also see the type of spores, which may not be stained.
5. Note the morphology and staining character of given culture.

Result:



(Fig: Gram-positive rods)

(B) Cultural characteristics

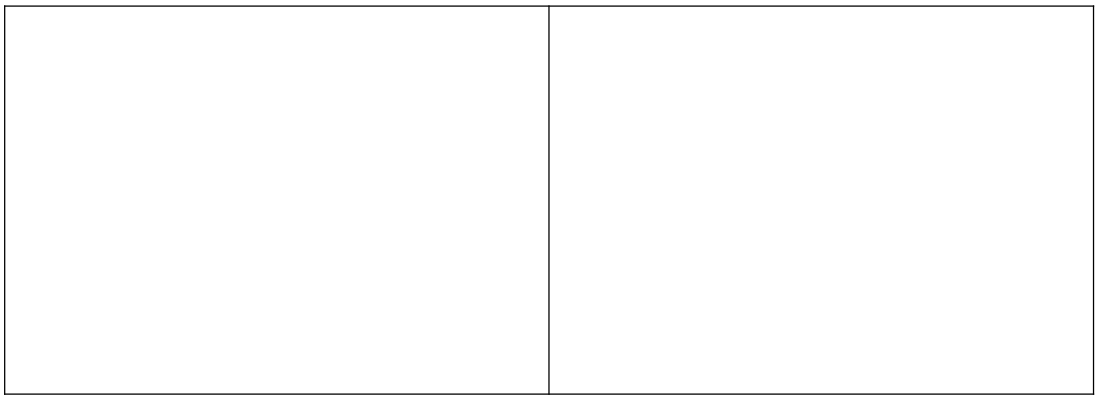
1. Inoculate the given culture in sterile nutrient broth with the help of sterile platinum loop to see pigment production, turbidity and pellicle formation, it any.
2. Streak the sterile nutrient agar, blood agar and Robertson cooked meat media plates and infusion broth with the given culture to see the colony characters and other differential features of the organism including haemolysis.
3. Stab the TSI slant with the culture and also spread the culture over the slant to see the acid/alkaline reaction and H_2 S production by the organism.
4. Incubate the broth clant and plates at 37^{O} C for 18 - 24 hours in Novy‟s or MacCantosh jar anaerobically.
5. Record the growth characteristic and other differential features.

Result/Observations:

Sl. No.	Media	Observations
1	Growth in nutrient both (Anaerobically)	
2	Growth on nutrient agar plate (Anaerobically)	
3	Growth in Robertson cooked meat infusion broth (Anaerobically)	
4	Growth in Robertson cooked meat agar plate (Anaerobically)	
5	Demonstration of motility by cavity slide method	

6	Growth on blood agar plate (Anaerobically)	

(C) Motility: A drop of broth culture is put on a cover slip and examined for motility by hanging drop method in a cavity slide. Alternatively, the culture may be stabbed in the media containing semisolid agar and incubated at 37^{o} C for 24 hrs. Observe for spreading growth on either side of the stab.



Cavity slide method Stab culture method

(D) Biochemical characteristics

1. Inoculate the given culture in the following media for performing various biochemical tests.
2. Incubate the tubes at 37^{o} C for 18 to 24 hours under anaerobic conditions.
3. Note IMViC pattern and other differential features of the given bacteria

Results/Observations:

Sl. No.	Name of Media	Biochemical tests	Results
1	Peptone water	Indol test	
2	Glucose phosphate peptone water (GPPW)	MR test	
3	Glucose phosphate peptone water (GPPW)	VP test	
4	Koser‟s citrate agar slant	Citrate test	
5	Triple sugar iron (TSI) agar	For H_2 S	(d) Type of growth on slant

	slant	production	(e) Type of growth on butt (f) H_2S production
6	Growth in gelatin medium	Gelatin liquefaction	
7	Nitrate medium	Nitrate test	

(E) Sugar fermentation reactions:

1. Inoculate the given culture in the following 1% sterile sugar media tubes containing Durham"s tube (for gas production).
3. Incubate the inoculated sugar tubes at 37^{o} C for 24 to 48 hours and note the fermentation reaction (acid or alkali) and production of gas by the given culture.

Result/Observation:

Sl. No.	Name of sugar	Result (acid/alkali)
1	Glucose (anerobically)	
2	Lactose (anerobically)	
3	Sucrose (anerobically)	
4	Gas from glucose	
5	Litmus milk	(a) Acid (b) Digested

(F) Conclusion

Bovine Mastitis

Mastitis, an inflammatory condition of the udder or the mammary gland of the animals, is seen in all species of milch animals but is of special significance in dairy cattle and goats. Mastitis is a descriptive term indicating diverse abnormal changes in the mammary gland. It is characterized by tissue damage and inflammatory reaction leading to abnormality in the secretory products of the tissue. It is mainly caused by bacterial infections of the udder, though other factors like physical factors like mechanical injuries may predispose the udder to mastitis. It may be acute, sub-acute or chronic or even sub clinical depending on the severity of the infection. One of the important organisms causing acute mastitis in cattle is *staphylococcus aureus.*

Bovine mastitis is one of the most important diseases of the high yielding cattle and buffaloes. It reduces the milk yield and fat content and affects the quality of the milk, thus putting the owner to a big economic loss. It is a problem causing huge losses to the national wealth throughout the world. Various organisms have been isolated from the animals suffering fro mastitis, viz., *staphylococcus aureus, streptococcus agalactiae, streptococcus dysgalactiae, E.coli, Salmonella spp., Pseudomonas aeruginosa, Klebsiella pneumonia, Corynebacterium pyogenes, Clostridium perfringens, Brucella aborutus, Pasteurella multocida, Bacillus spp., Proteus spp., Mycoplasma spp., Rickettsial spp*. Many fungi like *candida albicans* and *Rhizopus spp*. have also been isolated from animals suffering from mastitis.

Treatment of mastitis is becoming more and more difficult due to indiscriminate use of antibiotics, emergence of drug resistant bacteria, poor management and unhygienic conditions at place where milch animals are kept. The immunization is ineffective due to number of causative agents involved in mastitis.

Exercise No. – 19

Experiment: To study the isolation and identification of microorganisms from mastitis milk.

Sample: Clinical sample of milk of cow suffering from mastitis.

Materials:

1. Bacteriological slides, Bunsen burner/spirit lamp, bacteriological loop, test tubes cavity slide
2. Anaerobic jar (Novy‟s or MacIntosh and Fildes‟ Jar), hydrogen gas cylinder
3. Gram‟s staining set
4. Media: Nutrient broth, nutrient agar, blood agar, SM 110, Brilliant green agar, MacConkey lactose agar TSI media, (broth & solid media)
5. Media for biochemical tests and reagents, methylene blue

(A) Collection of milk sample from cow

1. Wash the udder of the cow with clean water and swab/dry the udder by clean cloth.
2. Discard few strips of the milk from the affected quarter of udder and collect 2 to 3 ml milk in sterilized test tube.
3. Bring the milk in the bacteriological laboratory.
4. Inoculate the milk samples on various culture media in the laboratory within 1-2 hours after collection.

(B) Isolation of microorganisms from mastitis milk

1. Shake the milk sample and inoculate the sterilized blood agar (BA), nutrient agar (NA plates and other medial as above mark with marker on the back of plate indicating the number of animal and affected test (RF, RH, LH or LF), as the case may be.
2. Incubate the plates at 37^{o} C overnight aerobically for the development of bacterial colony if any I the collected mastitis milk sample.
3. The isolated colonies of the bacteria thus obtained are maintained on nutrient agar slant for further identification of the bacteria.

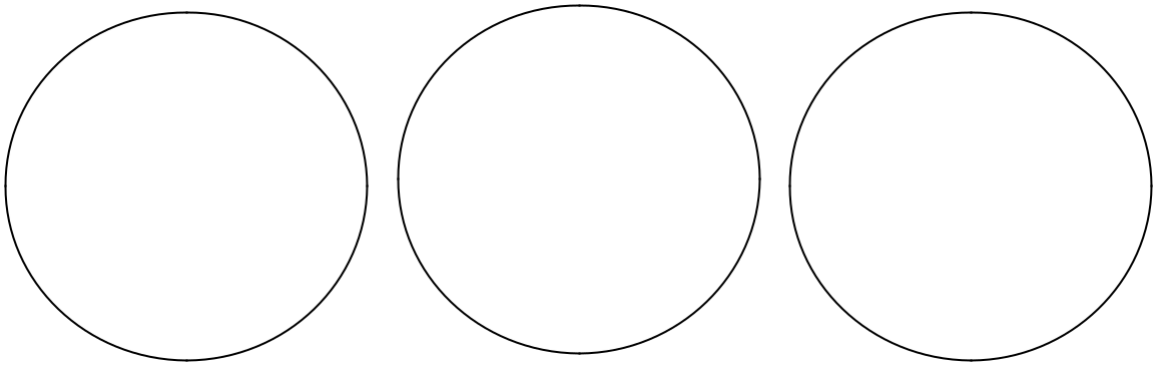


Fig. _____ Fig._____ Fig. _____

(C) Identification of bacteria from mastitis milk

(a) Morphological characteristics

1. Take a grease-free slide and mark tow lines on the slide at least one inch apart with the help of glass marking pencil.
2. Make the smear of given culture and fix it by passing the slide gently over the flame.
3. Stain the smear with gram‟s staining and remove the excess of moisture by blotting.
4. Air-dry the smear and examine under oil immersion objective.
5. Note the morphology and staining character of given culture.

Result:

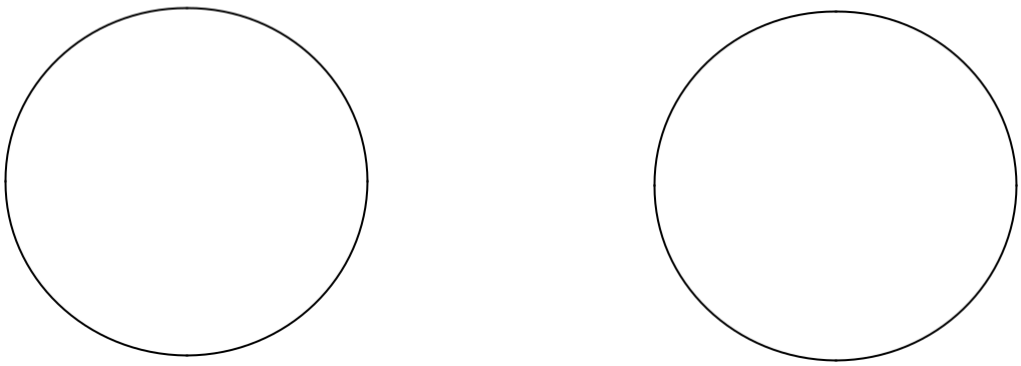


Fig: Gram-positive cocci in grape-like clusters **Fig:** Gram negative/positive rods

(b) Cultural characteristics

1. Inoculate the given culture in sterile nutrient broth with the help of sterile platinum loop to see pigment production, turbidity and pellicle formation, it any.
2. Streak the sterile nutrient agar and blood agar with the given culture to see the colony characters and other differential features of the organism.
3. Stab the TSI slant with the culture and also spread the culture over the slant to see the acid/alkaline reaction and H_2 S production by the organism.
4. Also inoculate the urea slant with the given culture.
5. Incubate the plates and tubes at 37^{o} C for 18 - 24 hours and record the results.

Result/Observations:

Sl. No.	Media	Observations
1	Growth in nutrient both	
2	Growth on nutrient agar plate	
3	Growth in blood agar plate	
4	Type of haemoolysis on blood agar plate	
5	Growth on MLA plate	
6	Growth on BGA plate	
7	Growth on TSI media	

(c) Biochemical characteristics

1. Inoculate the following media with the given culture isolated from mastitis milk of cow by sterile platinum loop for performing following biochemical tests.
2. Incubate the tubes at 37^{o} C for 18 to 24 hours.
3. Note the results.

Results/Observations:

Sl. No.	Name of Media	Biochemical tests	Results
1	Glucose phosphate peptone water (GPPW)	MR test	
2	Glucose phosphate peptone water (GPPW)	VP test	
3	Peptone water	Indol test	
4	Nitrate agar slant	Nitrate reduction test	
5	Rabbit plasma (1:5)	Coaglulase test (a) Tube test (b) Slide test	
6	Gelatin slant	Gelatin liquefaction	
7	Tetramethl-p-phenylene diamine redox dye	Oxides test	
8	Growth on urea agar	Urea test	
9	Ammonia production	Ammonia test	

(D) Catalase test

Take a drop of 3% hydrogen peroxide solution (freshly prepared) and pour it on a slide containing the culture or directly on the culture plate. In positive cases, the bubbling is seen due to breakdown of H_2O_2 by catalase enzyme produced by bacteria

$$2H_2O_2 + O_2 \xrightarrow{\text{Catalase}} 2H_2O_2 + O_2 \uparrow \text{bubbles}$$

(E) Sugar fermentation reactions:

1. Inoculate the given culture in the following 1% sterile sugar media tubes.
2. Incubate the inoculated sugar tubes at 37^{o} C for 24 to 48 hours and note down the results in terms of fermentation of sugar by the given culture (acid or alkali).

Result/Observation:

Sl. No.	Name of sugar	Result (acid/alkali)
1	Glucose (aerobically)	
2	Glucose (anerobically)	
3	Manitol (aerobically)	
4	Manitol (anaerobically)	
5	Lactose (aerobically)	
6	Sucrose (aerobically)	

(F) Conclusion

TUBERCULIN AND JOHNIN TESTS

The tuberculin and johnin tests are used for screening the cattle herds for tuberculosis and paratuberculosis, respectively. The two tests are based on the delayed type hypersensitivity reaction. Several tuberculins are used and all contain mycobacterial proteins, to which animals may be hypersensitive. Koch‟s old tuberculin, which has been used widely for the standardization of tuberculin‟s, is a filtrate of an 8-week old culture of *Mycobacterium tuberculosis*. Purified protein derivative (PPD) obtained from *M. bovis* is relatively pure tuberculin and because of its greater specificity and ease with which it can be standardized, it is widely used in the diagnosis of tuberculosis in cattle and buffaloes. A dose rate between 5000 and 10,000 tuberculin units (0.1 ml tuberculin containing 1 or 2 mg of bovine PPD) is considered to be most suitable. Avian tuberculin is also used in the comparative test (double intradermal) in cattle as well as in swine and poultry. The johnin is similarly prepared from m. paratuberculosis and used in the same way with same dose and route of inoculation.

In tuberculin test, where the johne‟s disease or avian tuberculosis is suspected or „skin tuberculosis‟ is apparent, non-specific sensitization must be excluded by a comparative test. The comparative test depends on the greater sensitivity to homologous tuberculin or PPD and johnin. Both are injected simultaneously into two separate sites on the same side of the neck 12 cm apart and one above the other. The test is read 72 h post-inoculation.

Exercise No. – 20

Experiment: To perform the tuberculin and johnin tests for screening/diagnosis of tuberculosis and johne"s diseases in cattle heard.

Materials:

1. Mammalian tuberculin (PPD), johnin
2. Vernier calipers, scale, razor and blade, cotton
3. Normal saline solution (0.85%), 70% alcohol

Method:

1. Shave 3 sq cm area of the neck (at two places 12 cm apart with the help of razor) of the animal suspected for tuberculosis and johne"s disease.
2. Care should be taken not to inflict injury/cut while shaving the area of neck.
3. Measure the skin thickness of both the shaved areas of neck with the help of vernier calipers.
4. Note the thickness of skin in mm.
5. Inject 0.1 ml of PPD and johnin with the help of tuberculin syringe simultaneously into two separate sites on the same side of neck, 12 cm apart and one above the other.
6. Care must be taken in placing the injections as sensitivity varies from place to place in the skin.
7. After 72 h of first injection, note the increase in thickness of skin (in mm) of both areas (if any) with the help of vernier calipers.
8. Note the type of swelling of shaved area (diffuse, hot, hard, painful and red).
9. Note the difference in the skin thickness of 1^{st} and 2^{nd} reading of the animal and observe the result as below.

Results

1. If the difference of the 1^{st} and 2^{nd} reading of skin thickness of neck region of animal is more than the double of 1^{st} reading and the type of swelling is diffuse, hot, painful and red at the site of PPD inoculation and not in the area where johnin was injected, the animal is tuberculin positive.
2. It the swelling is noticed at both the sites of neck region where PPD and johnin were injected and the swellings were similar as described above, the animal is johnin positive, johnin cross-reacts and dominates PPD reaction.

AGGLUTINATION TESTS

Agglutination test is one of the most widely used serological tests for the identification of microorganisms. In this test, we use particulate antigen (bacterial antigen) and the test is performed in small serological tubes (75 x 9 mm size). The procedure consists of mixing suspension of the microorganism (agglutinate) with serum containing antibodies (agglutinin). Dilutions are made in normal saline solution (0.85% sodium chloride) with graduated pipette marked with a pencil. Automatic pipettes or pipettes can be used if the number of samples to be tested is more. Mixture of antigen and antibody is incubated at 45^{o} C in water bath and clumping of bacterial cells is noticed. Serological reactions are highly specific and widely used for the identification of bacterial species or „Types" within a species.

Exercise No. – 21

Experiment: To perform tube agglutination test for the diagnosis of brucellosis in cattle

Materials:

1. Normal saline solution (0.85% sodium chloride), heat-killed suspension of bacterial cells (antigen; washed saline suspension of *Brucella abortus*), normal serum and antiserum.
2. Serological tubes (75 x 9 mm size) and test tube stands, one ml pipettes.
3. Water bath

Method:

1. Arrange 10 serological test tubes in a serological rack and label them 1 to 10.
2. Add 0.8 ml of physiological saline solution (freshly prepared) to the first tube and 0.5 ml to all the rest 9 tubes as shown in the protocol given below.

Protocol

	Tube Number									
	1	2	3	4	5	6	7	8	9	10 Antigen control
Normal saline (ml) (0.85%)	0.8	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Antiserum (ml)	0.2									
Transfer to next tube(1 to 8)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5*	
Antigen (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

(TITRE) reciprocal of final serum dilution	10	20	40	80	160	320	640	1280	2560	

* discard 0.5 ml

3. Add 0.2 ml of antiserum /unknown serum in the first tube by using a clean sterile pipette.
4. Mix the contents of the first tube by gentle shaking and transfer 0.5 ml from the first tube to the second tube by separate pipette.
5. Mix the contents of the second tube as before and transfer 0.5 ml of the 3rd tube.
6. Continue this procedure up to the 9th tube.
7. Discard 0.5 ml from the 9th tube, since 10th tube serves as control for antigen suspension.
8. Add 0.5 ml of known antigen (known) to each tube.
9. Incubate the serological tubes along with rack in a water bath at 45 C for 2-4 h.

Results

Observe each tube individually with care for evidence of clumping/agglutination. Find out the end point and calculate the agglutination titre.

Exercise No. – 22

Experiment: To perform slide agglutination test for on the spot diagnosis of brucellosis in cattle

Materials:

1. Bacteriological loop, glass microscopic slides, 13 x 100 ml test tubes, sterile 1 ml pipette, applicator stick
2. Washed saline suspension of coloured *Brucella abortus* antigen.
3. Physiological saline solution (0.85% sodium chloride) and antisera.

Method:

1. Divide the slide into two equal parts and label the parts as A and B.
2. To area A, add one drop of coloured *Brucella abortus* antigen and one drop of 0.85% normal saline solution with the help of pipette.
3. To area B, add one drop of coloured *Brucella abortus* antigen and one drop of *Brucella abortus* antisera.
4. Mix the contents in area A and B with the help of applicator stick or tooth pick.
5. Take the slide with two fingers of one hand and move the slide back and forth in order to mix the contents.
6. Observe the slide macroscopically as well as microscopically under low power microscope for cellular clumping or agglutination.